NEWCASTLE DISEASE VACCINES

Newcastle disease has caused, and continues to cause, very heavy losses to the poultry industries of many countries. In a number of developing countries, the high prevalence of the disease seriously impedes the development of poultry production. There are, however, effective methods of controlling the disease. Vaccination is a key measure, but for this procedure to be effective it is essential to produce high-quality vaccines and to apply them properly.

This publication covers all aspects of Newcastle disease immunization, discussing forms of the disease and methods of spread; types of vaccine available; production, testing and application of vaccines; and vaccination programmes. It is hoped that it will be useful in the establishment of Newcastle disease vaccine production units, and to those engaged in poultry disease control programmes.

COVER: Harvesting vaccine.
Photo by Bella Jenö, Budapest, Hungary.

NEWCASTLE DISEASE VACCINES: THEIR PRODUCTION AND USE

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THEIR PRODUCTION AND USE

by

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PREFACE

Newcastle disease remains a hazard to the poultry industry of many countries. Both sanitary measures and the use of vaccines are needed to control it.

The increasing problems associated with Newcastle disease control have emphasized the need to examine the preparation and application of different vaccines, and it is hoped that the techniques described in this publication will be useful in the establishment of Newcastle disease vaccine production units.

Other sources of information on Newcastle disease are *Newcastle disease virus: an evolving pathogen* (1964), published by the University of Wisconsin Press; *Newcastle disease: a review 1926-1964* (1966) and *Newcastle disease: virus and spread* (1975), both published by the Canada Department of Agriculture; *Methods for examining poultry biologics and for identifying and quantifying avian pathogens* (1971), published by the National Academy of Sciences of the United States; and *Isolation and identification of avian pathogens* (1975), published by the American Association of Avian Pathologists.

This publication is a revised edition of *The production and use of Newcastle disease vaccines*, published by FAO in 1973.

Appendix 1 gives definitions of certain terms used in the text.

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INTRODUCTION

Newcastle disease affects both domestic and wild birds, and has become a major problem in many countries where poultry are reared. The original descriptions by Doyle (1927) and Kraneveld (1926) related to the disease in its virulent form. By contrast, Beach (1944) showed that the disease termed avian pneumoencephalitis was caused by a mild strain of the virus.

Following the widespread recognition of Newcastle disease, control measures involving slaughter, sanitary measures and vaccination were adopted by national disease control agencies. These measures have had varied success, and the progress made in a number of countries up to 1964 has been reviewed by Lancaster (1966). The systematic vaccination of all poultry in a highly infected area has given very satisfactory results. For example, in Hungary the acute form of the disease, very widespread after the Second World War, was almost eradicated after all the poultry in the country were vaccinated during a three-month period (Benedek and Tóth, 1950). However, in 1967-68 the disease became very difficult to control in several countries of the Near East and the eastern Mediterranean, and in 1970 a serious epidemic occurred in Europe (United Kingdom, 1971; Chu, 1971).

The severity of the disease in any one country does not remain constant, and from time to time isolates occur which are of greater virulence and necessitate a different control programme.

Newcastle disease is highly contagious and attempts to control it by veterinary sanitary measures alone are often unsatisfactory. Where the disease has become endemic, systematic vaccination of the total poultry population is a successful method of control.

While the origins and epidemiology of the disease may be obscure (Hanson, 1972), its impact in recent years has resulted in the need for vaccination programmes using vaccines which are highly effective and which cause minimum reaction in the bird. Intensified vaccination programmes have brought clinical Newcastle disease under control.

In the section dealing with seed virus (Chapter 3), great importance is attached to the need for master seeds to be pure and in a sufficiently immunogenic state to produce effective vaccines. The techniques required for passaging the virus are straightforward. However, the facilities required for the production of specific pathogen-free stock and for con-

trolled test units may be more difficult to achieve. In these circumstances, it may be advantageous to obtain a seed virus which is known to be in a satisfactory state for vaccine production, rather than conduct this developmental work locally.

1. THE VIRUS

The Newcastle disease virus is a paramyxovirus. Its shape is more or less spherical, although pleomorphic forms are also common. Typical virus particles are between 100 and 300 millimicrons in diameter (Waterson and Cruickshank, 1963). The envelope contains the myxovirus fringe consisting of haemagglutinin and neuraminidase protein. Below the projections on the envelope there is a layer of lipid, and the use of lipid solvent causes the disaggregation of this layer and the disruption of the virion.

The internal component of ribonucleoprotein forms a symmetrical helix which has a periodicity of about 17 millimicrons. The virus is an RNA virus and is single-stranded. Genetically it is thought to be either diploid or polyploid (Granoff, 1964). Purified RNA is not infective and is found as one strand with a molecular weight of about $10^{7.0}$.

The virus is readily inactivated by formalin, alcohol, merthiolate, lipid solvents and lysol. It is thermolabile, and most strains are fully inactivated by a 30-minute incubation at 60°C. Thermostability varies, and the resistance of different strains or substrains to heat is one of the most useful genetic markers available.

All strains grow in embryonating eggs and isolates do not require adaptation. The most common route of inoculation is the allantoic sac, in which the virus can usually be grown to a titre of about $10^{9.0}ELD_{50}$ (embryo-lethal dose 50 percent) per 0.1 ml.

Lentogenic virus strains take longer to kill the embryo, but produce significantly higher titres than the velogenic strains which kill the embryo in a much shorter period. The haemagglutination (HA) titre of intact virus in allantoic fluid varies from 2 000 to 8 000 HA units per 0.2 ml. Virus that has been disrupted by sonication or by lipid solvent treatment will haemagglutinate at a much higher titre due to the breakdown of the virus envelope to subvirus rosettes. This disrupted virus is not used for HA or haemagglutination inhibition (HI) tests because its tendency to aggregate into clumps renders the titre unstable.

The virus titre can also be estimated by assay of the haemagglutinin. This method is not precise because it does not distinguish between infective and non-infective particles, nor between whole virus or virus subunit rosettes.

The virus can be grown in a variety of cell culture systems, the most

commonly used being the chick embryo fibroblast monolayer (Roman and Simon, 1976), the chicken kidney cell monolayer, and baby hamster kidney (BHK) cells, either in monolayer or in suspension culture.

The titre of the virus in cell culture systems is usually 1 log lower than the corresponding titre in embryonating eggs.

Avian hosts

The Newcastle disease virus has been recovered from a variety of avian species. The disease is seen most frequently in domestic poultry including guinea-fowl; these species are more susceptible than the turkey and peafowl. Ducks, geese, partridges and quails are relatively resistant. In pheasants and pigeons the virus can cause severe disease, usually of the nervous type. The virus in its virulent form is lethal to many different species of birds, and isolates have been made from naturally infected psittacine species (United Kingdom, 1971).

Antigenic composition of Newcastle disease virus

Numerous authors have reported serological differences between isolates of Newcastle disease virus (Alexander and Allan, 1973; Gomez-Lillo *et al.*, 1974; Schloer, 1974; Schloer and Hanson, 1971; Schloer *et al.*, 1975; Spalatin and Hanson, 1976; and Waterson *et al.*, 1967). These differences have suggested that there might be a need for different strains of vaccine for specific types of virulent field virus. It has been reported that antigenic variation may be an *in vitro* finding only, and therefore of no significance in deciding the choice of vaccine strains. However, due to the importance of this problem the Central Veterinary Laboratory at Weybridge, United Kingdom, collected 14 strains of virulent virus from widely different geographic locations. A number of these strains had been reported as showing apparent antigenic variant behaviour on the basis of serious disease losses in vaccinated poultry.

Monospecific antisera against each strain were produced in specific pathogen-free (SPF) birds in isolators by injecting groups of 10 six-week-old birds with 0.5 ml of formalized amnio-allantoic fluid (AAF). Six weeks later, a dose of 10^9 EID_{50} (embryo-infective dose 50 percent) of the virulent virus was injected. Sera were collected at two weeks and 10 weeks after challenge. These were used as specific antisera.

The cross HI tests were conducted using the constant-virus varying-serum method (the beta HI test) with carefully adjusted 4 HA units per antigen. The results showed evidence of a serological variation between strains.

The results of plaque reduction tests carried out on chick kidney cell

TABLE 1. — RESULTS OF PLAQUE REDUCTION TESTS

Antigen	Sera								
	North. '72	Essex '70	Paraguay	Finland	Vom OSB 1	Vom OSB 2	Herts '33	La Sota	B1
	log_2								
North. '72	6.77	6.14	8.22	7.26	6.41	7.68	6.66	5.61	7.46
Essex '70	8.14	7.45	8.54	7.47	6.27	7.50	6.90	5.89	8.01
Paraguay	6.74	5.94	7.89	6.35	6.31	7.50	8.11	5.75	7.45
Finland	7.10	6.37	6.95	7.73	6.16	7.27	7.22	5.42	6.88
Vom OSB 1	5.92	5.54	7.85	6.05	7.49	7.85	6.54	5.21	5.70
Vom OSB 2	7.18	6.59	7.69	7.10	6.21	8.13	7.44	6.34	7.37
Herts '33	6.87	6.02	7.45	6.79	5.50	7.48	6.77	5.63	7.01
La Sota	6.99	6.85	7.79	6.98	6.59	7.12	6.90	5.98	7.28
B1	7.03	6.54	7.40	7.26	6.14	7.48	6.78	5.73	7.29

TABLE 2. — ANTIGENIC RELATIONSHIPS (TRANSFORMATION OF PLAQUE REDUCTION TITRES[1])

Antigen	Sera							
	North. '72	Essex '70	Paraguay	Finland	Vom OSB 1	Vom OSB 2	Herts '33	La Sota
North. '72								
Essex '70	1.029							
Paraguay	1.100	1.348						
Finland	1.104	1.586	2.245					
Vom OSB 1	1.950	1.943	1.534	2.838				
Vom OSB 2	1.010	1.678	1.334	1.679	1.719			
Herts '33	1.002	1.566	1.363	1.174	2.170	1.008		
La Sota	1.050	1.274	1.122	1.509	1.788	1.251	1.087	
B1	1.413	1.065	1.225	1.362	2.763	1.219	1.100	1.096
Mean value	1.199	1.570	1.439	1.574	1.767	1.344	1.273	1.405

[1] Archetti and Horsfall transformations of plaque reduction titres of monospecific sera tested against 100 ± 30 PFU of test virus. The log figures from Table 1 are expressed as their % activity in relation to the mean of all tests for that serum in order to adjust the values for the variation in potency between sera. The Archetti and Horsfall transformation using non-logarithmic serum end points was:

$$\sqrt{\frac{\text{Heterologous 1}}{\text{Homologous 1}} \times \frac{\text{Heterologous 2}}{\text{Homologous 2}}}$$

All products less than 1.00 were expressed as the reciprocal.

cultures using approximately 100 plaque-forming units (PFU) per dish and doubling dilutions of serum are shown in Table 1.

The antigenic relationships were calculated by the method of Archetti and Horsfall (1950) from the plaque reduction tests in the table. These data, shown in Table 2, confirm previous studies indicating that serological variation occurs between strains of Newcastle disease virus.

The results reported above indicated that further information would be obtained from a cross-protection test. For this experiment, groups of 3-week-old susceptible chicks were used for testing the variant strain Vom OSB 1, the reference strain Herts '33 and the vaccine strain B1. The results of the cross-protection tests are shown in Table 3. The interpretation made is that full cross-protection exists between the Herts '33 strain and the Vom OSB 1 strain, and that the B1 vaccine is equally effective in protecting against both the virulent strains.

TABLE 3. — RESULTS OF CROSS-PROTECTION TESTS

Vaccines used	Challenge virus used			
	Vom OSB 1		Herts '33	
	HI titres	*Mortality*	*HI titres*	*Mortality*
Vom OSB 1 vaccine (inactivated)	[1] $2^{8.25}$	0/44	$2^{6.93}$	1/45
Herts '33 vaccine (inactivated)	$2^{8.48}$	1/45	$2^{5.96}$	0/45
B1 vaccine (live)	$2^{9.93}$	0/45	$2^{7.08}$	0/45
Controls		5/5		5/5

[1] Post-challenge HI titres

The conclusion made from this study as shown in Tables 1, 2 and 3 is that with the strains used, antigenic variation occurs as a serological phenomenon, but the cross-protection tests failed to confirm that the *in vitro* results are significant in field vaccination (Kaliannan *et al.*, 1975; Martone *et al.*, 1976).

2. THE DISEASE

Occurrence

Newcastle disease occurs in every continent of the world with the possible exception of Antarctica. It is enzootic in many countries, and severe outbreaks have occurred in areas where poultry production is intensive and where large numbers of poultry are reared on a single site or premises. Also, the location of sites near a packing plant or processing station often involves the movement of personnel and vehicles, thus creating conditions for large-scale outbreaks of the disease. Situations of this kind can lead to greater disease problems than husbandry methods involving smaller units and less communication between farms or premises. However, in some countries where household or backyard poultry keeping is common, birds may be on free range, and in this situation Newcastle disease can spread easily. Systematic vaccination in the whole area or region has given satisfactory results (Benedek and Tóth, 1950). A high density of poultry in an area, the use of forced ventilation in poultry houses, the transportation of live birds and the removal of litter to agricultural land all combine to facilitate the spread of the disease.

Effective disposal of litter and infected birds by burial or incineration is necessary to control the disease. The control of the movement of birds out of an infected area has also been of value. The possible spread of the disease must be anticipated where poultry are kept under semi-intensive conditions and where wild birds have access to poultry food and the poultry range. Similarly, the movement of hatching eggs or day-old poultry, contaminated cages and vehicles from an infected premises or area constitutes a high risk of spreading the disease.

The location of poultry breeding units in areas of broiler production will tend, under epizootic conditions, to cause a high rate of Newcastle disease infection among breeder birds. If these are well vaccinated, the losses from disease, both in terms of death and of lowered production, may be slight. What may be of greater importance is the effect of the disease on the antibody levels in the offspring, making active immunization of day-old chicks difficult or impossible.

Forms of the disease

Newcastle disease virus has been classified according to virulence for chickens. Strains are generally designated as belonging to one of three types:

Type	MDT [1]	ICPI [2]	IVPI [3]	Lesions
Lentogenic	96-168	0.0-0.4	0.0	Slight congestion in young birds
Mesogenic	44-70	0.4-1.9	0.0-0.5	Congestion
Velogenic	40-70	2.0-3.0	0.5-2.8	Severe lesions

[1] Mean death time (hours in eggs). — [2] Intracerebral pathogenicity index. — [3] Intravenous pathogenicity index.

Details of the above tests are given in Chapter 9.

The virus has also been classified by the main predilection sites, i.e., pneumotropic, viscerotropic or neurotropic (National Academy of Sciences, 1971). These terms refer to the greatest activity of a particular strain as shown by the presence of relatively large quantities of virus and marked clinical signs in a particular tissue. Thus, strongly pneumotropic strains produce marked respiratory lesions and may cause varying degrees of respiratory distress, with signs of paralysis in some birds. The degree of involvement of the respiratory tract is associated to some extent with the presence of other infectious agents including mycoplasmas and *E. coli.*

Viscerotropic strains may be of the mild avirulent type, e.g., Ulster 2C (McFerran and Nelson, 1971), or Queensland V4 (Simmons, 1967). Strains of this type are characterized not on the basis of signs or symptoms, but only on the fact that the virus can most easily be recovered from the intestinal tract and occurs at a much lower titre in the respiratory tract when compared with other strains. The most highly virulent velogenic virus is usually the viscerotropic type. This type causes marked haemorrhages in the proventriculus, and small haemorrhages or marked ulcers in the intestinal tract. The " Asiatic " form of the disease is now generally termed viscerotropic velogenic Newcastle disease virus (VVND). Not all birds infected with the latter virus will show typical symptoms (Alexander and Allan, 1974).

Most isolates from cage birds, especially psittacines, are of the viscerotropic velogenic type, and at the Central Veterinary Laboratory at Wey-

bridge, United Kingdom, these isolates have a range of intravenous pathogenicity index values from 1.9 to 2.8, with a median of 2.6, suggesting a virus of danger to other birds. Similar findings were reported by Chu *et al.* (1976).

The virus isolated in Iraq in 1968, in a number of other countries in the Near East, and in 1970 in western Europe, has been given the general designation Essex '70 to distinguish it from VVND isolates.

The Essex '70 type has exhibited more marked pneumotropic qualities than any other virulent examined to date. Although the intravenous pathogenicity index values have ranged from 2.2 to 2.6 with a median of 2.4 (see Tables 4 and 11), pneumotropic signs have predominated, and only a proportion of birds showed visceral ulcers typical of VVND. Clinically, the Essex '70 type of disease is characterized by very rapid spread from farm to farm and by acute respiratory distress. There has been evidence of windborne spread (Hugh-Jones *et al.*, 1973; Lancaster and Alexander, 1975). The Essex '70 type has not been associated with imported cage birds and therefore appears to be particularly adapted to domestic fowl. The pneumotropic qualities are not very stable in the isolated virus and decline after one egg passage, and the strong pneumotropic quality cannot be fully regained by direct bird-to-bird passage. However, the neurotropic and viscerotropic qualities of the virus appear to be more stable. Air samples from birds infected with pneumotropic virus have a considerably higher number of infected particles than air from viscerotropic infections.

Methods of spread

Methods of spread may be divided into two groups: those due to natural spread, and those due to mechanical factors associated with the transport of eggs, birds, carcasses, poultry offal, feed, vaccinating crews and the movement of personnel (Burridge *et al.*, 1975). In some instances, the disease has been introduced to other countries by frozen poultry meat. These and other aspects of spread of Newcastle disease have been discussed by Lancaster (1966), Hanson (1972) and Lancaster and Alexander (1975).

Eggborne virus will often kill the embryo during incubation, and field evidence has indicated that placing infected eggs in incubators has resulted in the spread of the disease. In addition, contamination of a hatchery can occur if an infected egg is broken. When infection of chicks occurs in the hatchery, the onset of symptoms may appear as early as 3 to 4 days of age, provided the maternal immune levels are low. If the immune levels are higher, overt disease may not be seen for a longer period.

Movement of growing stock may also be responsible for the spread of the disease. In the case of vaccinated birds, infection with Newcastle

disease virus may be subclinical, and the introduction of these birds to an unprotected flock may cause serious losses (Stone *et al.*, 1975). Disease may also be spread by the use of contaminated vaccines, and by untested or autogenous vaccines over which there has been no quality control testing.

The effective control of the disease depends equally on understanding the methods of spread, the quality of the vaccines, and their application.

3. SELECTION OF THE VACCINE SEED STRAIN

The selection of seed strains for vaccine preparation is of the greatest importance in the production of efficient and safe vaccines. At present there is no central repository of vaccine strains, although a repository of Newcastle disease strains is maintained at the University of Wisconsin in the United States, and at a number of other laboratories including the Central Veterinary Laboratory at Weybridge, United Kingdom. These stocks of freeze-dried virus are not specifically designed for use as master seed virus for vaccine production, and the strains may need to be passaged at limiting dilutions in SPF eggs, and also passaged through SPF chickens to regain a maximum infectious activity.

Limiting dilutions

A limiting dilution is one in which the volume of inoculum placed in any one egg has a high probability of containing only one virus particle. Table 5 shows a typical titration for seven embryos inoculated per dilution. Passage to limiting dilutions resulted in the obtaining of an isolate of low virulence from the virulent Essex '70 strain (Reeve *et al.*, 1974).

In this example, the dilution $10^{-7.7}$ ($\frac{1}{50\,000\,000}$) is not a limiting dilution because the end point was not determined. However, $10^{-8.4}$ and $10^{-9.1}$ are both limiting dilutions, although the $10^{-9.1}$ is preferable. The dilution $10^{-9.8}$ has no detectable virus in the seven inoculated eggs.

Virus infectivity

Virus that has been passaged many times in embryos or stored in the laboratory for a long time may lose some of its infectivity and ability to spread from one bird to another (Allan, unpublished results). As these features of a vaccine strain are important for mass vaccination techniques, such as application in the drinking water, it may be necessary to passage the seed virus through chickens three times to regain the factor of contagion. This may be done by splenic passage in which the recommended technique is to remove the spleen of a bird four days after it has been infected with at least 10^{7} EID$_{50}$ of the parent seed virus. Inoculation of a

TABLE 4. — CURRENT EPIDEMIC STRAINS FROM WORLD COLLECTION

Country	Code	ICPI [1]	IPVI [2]	MDT [3]
Iraq	AG 68	1.77	2.52	70
	AG 68 265	1.75	2.36	64
Lebanon	1970	1.88	2.61	48
Greece	1972	1.74	2.36	49
Israel	1972	1.89	2.63	44
France	Lille	1.77	2.49	48
Netherlands	4799/70	1.84	2.47	61
	66015/70	1.75	2.63	59
	66027/70	1.75	2.54	56
Canada	9380/71	1.76	2.43	51
	9379/71	1.50	1.94	70
	6469/71	1.81	2.31	54
	S1068	1.76	2.10	54
United States	NY 70181/72	1.77	2.59	51
	Texas 219/70	1.81	2.60	61
	Cal 1085/71	1.83	2.57	58
Paraguay	2098	1.76	2.52	57
Colombia	829	1.69	2.39	62
United Kingdom				
Essex	Lamb/70	1.73	2.24	63
Northampton	/72	1.91	2.75	53

Accepted challenge strains	ICPI [1]	IPVI [2]
GB Texas	1.75	2.66
Herts '33/'64	1.88	2.64
Herts '33/'56	2.00	2.71
Milano	1.86	2.81
Teramo CTE	1.96	2.76

SOURCE: Central Veterinary Laboratory, Weybridge, United Kingdom.
[1] Intracerebral pathogenicity index. — [2] Intravenous pathogenicity index. — [3] Mean death time (in hours).

TABLE 5. — TOTAL EMBRYOS DYING IN A VIRUS TITRATION (SEVEN EMBRYOS PER DILUTION)

Dilution								*Embryos dead*
$10^{-7.7}$	D	D	D	D	D	D	D	7/7
$10^{-8.4}$	D	D	D	D	D			5/7
$10^{-9.1}$	D	D						2/7
$10^{-9.8}$								0/7

supernatant of homogenized spleen into the second bird, and subsequently into a third, will usually produce a more infectious virus. For some strains of virus splenic passage may not be very useful, and similar passages using material from the trachea may be more successful. This passage technique is limited to regaining contagiousness, provided this characteristic was present in the original material (Eidson *et al.*, 1977). The technique depends on strict isolation facilities being available. In some cases, a virus strain has been sufficiently altered to prevent full recovery of its original virulence.

Laboratory studies with the lentogenic vaccine strains have produced some conflicting results upon comparison of strains. This variation probably represented the different passage histories which individual lines of virus strains have undergone since their origin. It must be emphasized that the common vaccine strains were developed before SPF propagation became standard practice. Also, different passage techniques, including dilution methods, incubation times and the number of passages made, have resulted in variation between different lines from an original virus strain.

The more powerful vaccine strains are listed together under the heading of mesogenic strains (Chapter 10). These viruses, like the lentogenic strains, were developed before the advent of SPF passage, and some of their characteristics have diverged since their origin.

The mesogenic Komarov strain has been widely believed to be noncontagious, and therefore would not spread from vaccinated to unvaccinated stock. This has been observed to be untrue in some instances. V. Sohrab (personal communication, 1973) and current studies at the Central

Veterinary Laboratory at Weybridge have shown that some seed strains of this vaccine are directly contagious and that others can be made contagious by selective passage in chickens.

Although Newcastle disease virus does not exhibit the full von Magnus phenomenon (von Magnus, 1951) whereby passage at high multiplicity (Appendix 3) results in loss of fully infective virus, nevertheless passage in this manner affects the titre and infectivity of the progeny virus. These changes may be permanent or may, in some cases, be reversible by a limited number of rapid passages in chickens.

High multiplicity

For the purpose of this publication, high multiplicity may be defined as the infection of a chicken embryo with more than $10^{4.0}$ELD$_{50}$ of virus. The term also means the infection of a cell culture system containing a known number of susceptible cells with a viral inoculum of more than one infective particle per cell. Hence a cell culture tube containing 10^6 cells and infected with 10^9 virus particles would have been infected with virus at a multiplicity of 1 000 particles per cell, that is 10^3.

Seed virus of low passage level

As most of the well-characterized vaccine strains are more than 20 years old, it may not be possible to obtain seed virus that is under 10 serial passages. However, selection of a new seed strain may prove difficult.

It is recommended that seed virus be obtained at as low a passage level as possible, and that on receipt the virus be subjected to limiting passage in fully SPF eggs before production of master seed. Institutes that have produced Newcastle disease vaccines may have carried out considerable work on their seed viruses before beginning vaccine production, and have produced lines of virus which are at their full potential. In many cases, these lines may be protected by patent law in the country of origin. Subinoculation from these lines should only be undertaken with the permission of the institute concerned. The laboratory should be asked to furnish a full passage history of any vaccine strain they are willing to provide for the use of others.

Production of the master seed

Known seed virus. The following procedure is suggested:

1. The virus is obtained and at least 100 ml allantoic fluid prepared from eggs inoculated with the highest dilution possible. Thus 25 embryo-

nating eggs are inoculated with a 10^{-5} dilution, and additional eggs with a lower dilution (higher virus concentration), in case the shipped ampoule has low viability.

2. The harvest is divided into 200 × 0.5 ml amounts and stored at —60°C or in freeze-dried ampoules. This will be designated " master seed passage 1 " by the receiving laboratory.
3. One hundred to 1 000 ampoules of working seed are prepared from the master seed, and these will be designated " working seed passage 2."
4. Production batches are prepared from the working seed. This method will allow the preparation of 100 to 1 000 production batches of vaccine (less those used for tests).
5. It is advisable to store the working seed in frozen state at —20°C or lower. Lyophilized seed virus in the first egg passage usually does not multiply to the desired titre of $10^{9.5}$ EID_{50}/ml. Therefore, it is usually necessary to make two or three consecutive egg passages from the lyophilized material to obtain a high virus titre. At intervals of every six to eight months, new working seed virus material should be prepared from the master seed virus material.

When the supply of ampoules of working seed is exhausted, a second ampoule of master seed passage 1 is used to prepare a further supply of working seed. Thus the original virus seed will last for 10 to 100 years.

Unknown seed virus. If the virus when received is not in an SPF state, or if it needs to be passaged in chickens before use, the passage levels will increase. The following procedure is suggested:

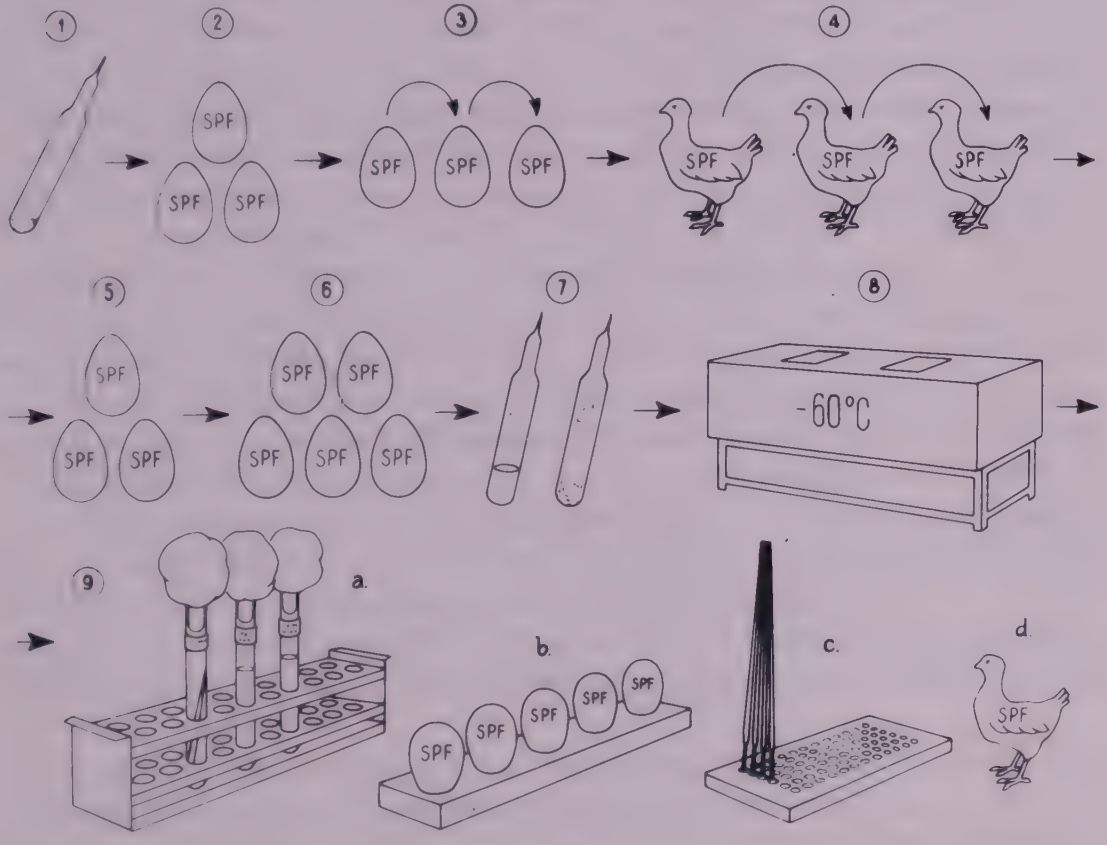
Passage 1. Propagation of the virus received in SPF eggs.
Passage 2. Limiting dilutions in SPF eggs
Passage 3. Limiting dilutions in SPF eggs
Passage 4. Limiting dilutions in SPF eggs
} Purpose: to produce limiting dilution material which can be considered genetically pure and free from contaminants.
Passage 5. Injection of SPF chicks with 10^{-7} dilution.
Passage 6. Injection of SPF chicks with 1:100 suspension of spleen or trachea from previous chicken passage.
Passage 7. Repeat above passage.
Passage 8. Production of active virus in allantoic fluid in SPF eggs. The content of virus in the spleen or trachea may not exceed 10^3 per 0.1 ml and hence the eggs should be inoculated at a dilution of 10^{-1} or 10^{-2}.
Passage 9. Production of a large batch of working seed from passage 8 by inoculation at 10^{-5} or lower.

Passage 10. Storage of seed virus.

This procedure is summarized diagramatically in Figure 1.

Comparison of strains. It is easier for a new laboratory to start with a known virus than to develop a new seed culture. This means that the receiving laboratory must have full confidence in the origin of the virus seed material. It must be recognized that comparative assay conducted

FIGURE 1. Flow diagram of the production of Newcastle disease vaccine
A. Production of master seed virus



Key to Figure 1

1. Original virus strain
2. Multiplication of the original virus strain in SPF eggs
3. Three consecutive passages in SPF eggs
4. Three consecutive passages in SPF pullets
5. Multiplication of the chicken-passaged virus in SPF eggs
6. Production of the master seed virus in SPF eggs
7. The virus is stored in undried, frozen and freeze-dried ampoules
8. Storage of master seed virus in a deep-freeze at —60°C
9. Sterility (a), safety and potency (b, c, d) tests of the master seed virus

with vaccines of different origins has not always given consistent results. A comparison of the immunogenicity of different vaccine strains may be conducted by determining the protective dose (PD). Thus Tóth and Markovits (1964) used the amount of virus (expressed as PD_{90}) which protected 90 percent of the vaccinated chickens. The PD_{90} for the B1 strain administered *per os* was $10^{5.27}$, and for the La Sota strain *per os* was $10^{4.71}$.

The following may be used as a guide to the efficacy and pathogenicity of different vaccine strains:

Lentogenic strains. The *F* strain has the lowest virulence of the common lentogenic vaccine strains. Respiratory reactions are minimal. The vaccine is most effective when given by an individual route (Mallick *et al.*, 1969).

The *B1* strain is slightly more effective and more virulent than the F strain in most comparative tests. The drinking water route is often used for mass administration of the B1 strain. The immune response in individual chickens depends on the handling of the subline of the virus used for seed virus production, and on the method of administration of the vaccine. The spray method of administration produces better immunity.

The *La Sota* strain is considered to be more pathogenic than either of the foregoing. Vaccination is often via the drinking water. This strain is more prone to give rise to respiratory complications and is of greatest use in flocks which are mycoplasma-free. It is often used as a booster dose in chickens which have received an effective primary dose of B1 or F vaccine. Reports on the efficacy of the La Sota strain have been variable, although it has generally been found that the boosting effect is more marked than with either of the two other milder strains. Bird-to-bird spread is only slight with the B1 vaccine, but effective spread occurs with the La Sota strain (Kreimer, 1969). The La Sota strain administered intraocularly or *per os* provided good immunity in turkeys (Winterfield and Fadly, 1973).

Mesogenic strains. The *Mukteswar* strain is the most invasive and therefore provides the greatest and most durable immunity. However, it is one of the most pathogenic vaccine strains, and its use is confined to growing birds which have been previously immunized with one or more doses of a lentogenic-type vaccine. The Mukteswar strain is used widely in tropical countries, particularly in Southeast Asia, and has been effective in giving a measure of control of the peracute type of the disease.

The *Hertfordshire* (H) and the *Komarov* (K) strains are less pathogenic than the Mukteswar strain, and may be combined with fowl pox vaccine. They are used in regions in which the disease is less acute and where the level of immunity has to be attained with the minimum of stress. The H strain is used by the subcutaneous or intramuscular route. This strain is

not administered *per os* because there is little or no spread between birds. The H strain is recommended for a booster vaccination of chickens over 8 weeks of age which have been immunized at an earlier age with a lentogenic vaccine (Papócsi and Tóth, 1973). The Komarov strain has also provided adequate immunity (Karczewski *et al.*, 1969).

The *Roakin* strain is used in a similar manner, but unlike those previously mentioned it is a naturally occurring isolate which has been attenuated (Zebrowski *et al.*, 1968). It has been used mostly in the United States.

The use of vaccine strains. The different vaccine strains developed in other countries in general correspond to those listed above (Malik and Dhawedkar, 1970; Sokkar and Sawa, 1974). Usually, the immunity provided by a live Newcastle disease vaccine is directly related to the degree of virulence. Thus the greater the required immunity, the more virulent the vaccine strain used. In general, mesogenic strains are not administered to poultry under 3 months of age, and usually are preceded by immunization with a lentogenic type of vaccine.

When Newcastle disease has become a problem in regions where it was previously controlled by vaccination, there is a tendency to assume that antigenic variation of the local field virus has occurred. Often the solution proposed is to develop a vaccine strain from the locally occurring field virus. Intensive analysis of numerous Newcastle disease isolates has failed to show any evidence of antigenic variation (Kendal and Allan, 1970).

Failure of the orthodox vaccine strains is more likely to be explained on the basis of faulty application and/or the poor quality of the existing vaccines, rather than any change in antigenic composition.

To avoid risks associated with the use of a virus which has not yet been established for use as a vaccine, it is suggested that the following procedures be conducted:

— Passage is carried out only in SPF eggs.
— At least three limiting dilution passages are undertaken to obtain a degree of genetic homogeneity.

These passages should be made by the injection of 0.1 ml of a dilution of at least 10^{-8} into a series of eggs, only a percentage of which will be infected. In this way, the chance of propagation from a single virus particle is increased, although the virus will still be genetically impure and unstable because of the diploid nature of the virion.

The safety of a new isolate can only be determined effectively in chicks which have no maternal antibody to mask the possible effects of the virus. The tests should include at least four passages in birds prior to the vacci-

nation of a larger number to observe lack of stress effects and to assay immunogenicity.

New vaccines of this kind should be subjected to repeated estimations of the mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) carried out on various passage levels (Chapter 9). These tests should be conducted before and after the final chicken passages in order to estimate any possible change in virulence.

The various passage levels should be stored, preferably at —70°C, in case attenuation becomes too extreme and it is necessary to re-examine a previous passage level.

Finally, the full range of safety tests for the possible presence of contaminating pathogens should be carried out before field evaluation is attempted (Chapter 7). Additional comments on the evaluation of a new vaccine have been published (National Academy of Sciences, 1971).

The risks of using seed virus of unknown history are:

— Contamination with other poultry pathogens.
— Contamination with other strains of Newcastle disease virus (Beard *et al.*, 1970).
— Genetically impure Newcastle disease virus which could result in small proportions of fully virulent virus being present in the vaccine.

Serum for purity testing of seed virus. A standard technique for the demonstration of the purity of a virus suspension is the neutralization of the specific agent (in this case Newcastle disease virus) by monospecific antiserum, followed by inoculation of the virus-antiserum mixture into eggs. This technique permits the growth and identification of any previously hidden pathogen. The serum used in this technique must be produced in SPF birds that have been tested and are known to be free of antibodies to contaminating viruses.

The virus neutralization test should be conducted with a low dilution (1:100) of the virus under test. The virus serum mixture should remain at room temperature for one hour. Newcastle disease sera used in this test should have a very high titre, not less than 2^{12}.

The serum should be diluted 1:4 prior to use and equal amounts of the virus and serum dilutions mixed together. The test should include falling dilutions of virus in the virus serum mixtures. The test should be controlled with normal sera. If the serum is used undiluted, it is possible that non-specific inhibition of a contaminant virus will occur, thus rendering the test valueless. If the virus preparation is diluted more than 1:100, it is possible that the contaminant virus will be diluted out. It is for these reasons that high-titre serum is needed to ensure effective neutralization of the main virus under test.

With the growing awareness of the importance of adenoviruses and unknown viruses in poultry diseases, it is now more important than ever to ensure that seed viruses are completely free from contamination. One way of achieving this is to neutralize the seed virus with pure monospecific Newcastle disease serum and then incubate the mixture on sensitive cell culture systems such as chicken kidney cell cultures. These tests are of value only if it is shown that the seed used to induce the response for the serum and the SPF birds for serum production are free from all known contaminants. The Central Veterinary Laboratory at Weybridge has produced limited amounts of Newcastle disease-specific serum for this purpose in 0.5-ml freeze-dried vials.

NOTE: Some recently identified lentogenic strains are of the previously viscerotropic types. A field trial with one of these isolates (Ulster 2C) showed it to be unduly sensitive to interference by maternal antibody and hence not effective under field conditions.

4. THE LABORATORY

Facilities

Vaccine production should be located in a building separated from diagnostic or research work in order to ensure complete isolation of the virus strains from possible contaminants. The building (Figure 2) should have separate rooms for the following production activities: an area devoted to the inoculation and harvesting of eggs; an area for the cleaning and autoclaving of infected material and equipment; an area for the production of clean sterile glassware; and a filling and testing area and storage facilities for vaccine in production and for the finished ampouled vaccine. In addition, the vaccine production unit should contain a control laboratory and a records office. Staff accommodation should be separated from accommodation used by workers involved with diagnostic or other pathogenic materials.

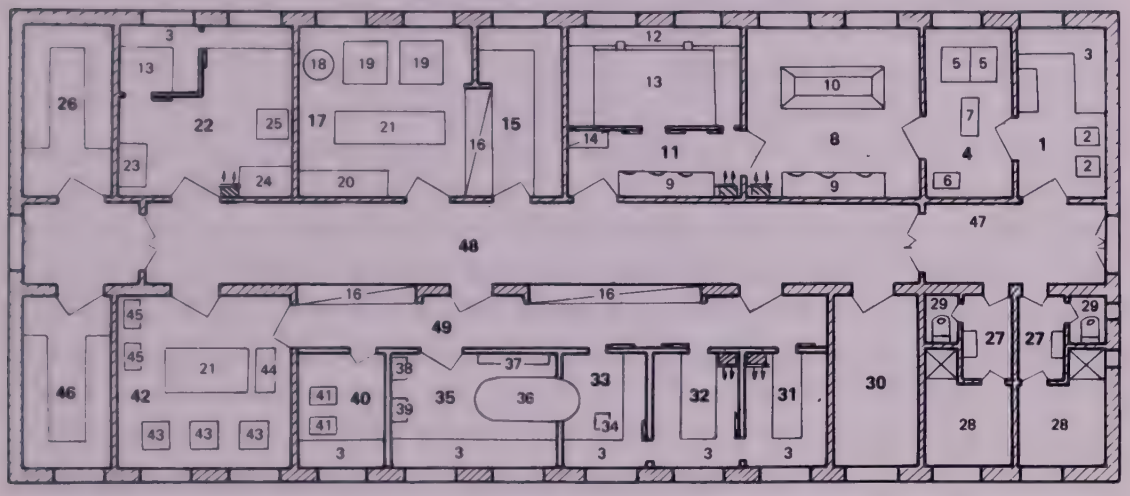
It is desirable to have filtered air in the production areas. This is most important for the areas used for the inoculation of embryos, the harvesting of embryo fluids, and the sterile filling of ampoules. Wherever possible, the control laboratory should also have air filtration.

The egg incubator should have ample capacity for the maximum number of eggs to be inoculated in any one week, and should be used for the production of only one type of vaccine at a time. It should be capable of being fumigated between batches of vaccine. Its temperature should be 37 $\pm$ 1°C. It should have adequate heating units for a rapid return to the working temperature after the doors have been closed. It is important that it be well insulated and have an automatic humidifier. The air circulation should ensure that all areas have the same working temperature. If uniform temperature is not achieved, then embryo death time will vary, resulting in an uneven harvest of virus. The temperature in different parts of the incubator should be measured and eggs should not be placed in areas of abnormal temperature.

As eggs are seldom sterile on the outside, the egg incubator can be a potent source of contamination, especially with moulds and bacteria. Fumigation of eggs prior to setting has considerably reduced the bacterial

contamination of the harvested fluids. It is highly recommended that each production batch of eggs be fumigated in the incubator before the embryos are inoculated. Fumigation with formaldehyde is preferred to egg dipping methods. It is recommended that the bacterial flora in the incubator be assessed at regular intervals by agar dish exposure methods or

FIGURE 2. Schematic arrangement of Newcastle disease vaccine production unit



Key to Figure 2

1. Egg storage room
2. Egg crate
3. Bench
4. Incubator and candling room
5. Incubator for 2 500 eggs
6. Incubator for 500 eggs
7. Candling table
8. Inoculation room
9. Table for setting and cauterizing of eggs
10. Hooded bench for inoculation
11. Harvest room
12. Aspiration
13. Sterile cabinet
14. Sterile gowns
15. Clean, sterile glassware room
16. Wall-case
17. Cleaning, autoclaving, sterilization room
18. Autoclave
19. Dry sterilizer
20. Washer
21. Preparing table
22. Control laboratory
23. Bench incubator
24. Deep-freeze (—60°C)
25. Refrigerator
26. Refrigerator room
27. Entrance room
28. Dressing room and shower } for men and women
29. WC }
30. Records office
31. Filling room
32. Stoppering room
33. Preparation
34. Recording instrument
35. Machine room
36. Freeze-drier
37. Spare parts case
38. Cutting of ampoules
39. Oxygen flame
40. Spare laboratory
41. Equipment in the spare laboratory
42. Testing room
43. Crimper
44. High-frequency equipment
45. Marking machine
46. Storage room
47. Air filters or laminar flow benches
48. Corridor
49. Clean corridor

other quantitative techniques. Should an increase in bacterial concentration occur, the incubator must be fumigated before production is resumed.

Eggs should be incubated in premises separated from the production area for nine days prior to candling and inoculation. If this is not possible, a separate egg incubator must be used. All infertile eggs should be removed before the batch is brought into the production area.

The egg inoculation room should have plain working surfaces preferably made of plastic laminates to ensure easy sterilization. It is advantageous to have the working surface protected by a hood device with ultraviolet strip lighting built into the hood to allow final disinfection by irradiation before inoculation is carried out. The inoculation room should be equipped with gas points for Bunsen burners and electrical outlets for an electric sterilizer for boiling syringes. Following inoculation, eggs should be sealed by paraffin wax or by collodion. If the latter is used, adequate ventilation must be provided and naked flames must be avoided to prevent fire risk from the inflammable solvents (Chapter 5).

Egg candling for the rejection of non-specific deaths and dead inoculated eggs prior to harvesting requires a room that is equipped in a manner similar to the inoculation room. This area should be capable of being blacked out to allow rapid candling of large batches of eggs.

The room used for inoculation may also be used for candling and the harvesting of allantoic fluid if the volume of vaccine being produced is limited.

Harvesting of virus fluids should be carried out under maximum conditions of sterility, and hooded areas above plastic laminated benching are recommended.

It is best to remove the allantoic fluids by suction provided by a pump drawing approximately 12.5 cm of water. This vacuum should be adjusted to the operator's convenience.

The harvest room should have ample provision for the disposal of used embryos. If possible, they should be autoclaved before final disposal. Containers used for this purpose should be sterilized before being introduced into the production area. Provision must be made for the sterilization of egg trays, and trays used for the incubation of infected eggs must be separated from similar equipment used for the incubation of healthy embryos. Four separate groups of egg trays may be necessary, and these should be identified.

Production security

All staff working in the production unit should be provided with sterile gowns which are changed daily. The staff should also be provided with

laboratory footwear which is kept in the production building. Head covering and latex gloves should be provided for the important operations of inoculation and harvesting of virus fluids. It is important that the staff employed in vaccine production not have casual access to any areas in which possible vaccine contaminants are handled. The staff must avoid contact with either commercial or experimental poultry units. Access to the production unit by visitors should be controlled at all times.

Virulent virus for challenge purposes must not be produced or handled in the production unit. The simultaneous production of avian vaccines other than Newcastle disease vaccine must be avoided. If necessary, these other vaccines can be prepared in the same facilities, but on another day. A thorough disinfection of the production unit must be conducted following preparation of each avian vaccine and of different strains of Newcastle disease vaccine virus.

The control laboratory should be provided with its own facilities for the storage of challenge virus in either liquid or freeze-dried form. Virulent virus should never be stored with vaccine production strains. Refrigerators operating at +4°C and —60°C are required. This laboratory should have a bench incubator for bacterial test media, and the working area should be sterile. The control laboratory will provide, on occasion, the virulent virus needed for the challenge of experimental birds. It is imperative that the work be arranged so that virulent virus is never handled on the same day as production material. Virulent virus should be reconstituted in a small separate area and the eggs used for propagation should be incubated in a bench incubator and never placed in the production incubator. All virus containers should be labelled with indelible ink.

As described for the production unit, the laboratory bench and surroundings should be disinfected on completion of work with virulent virus. All instruments and glassware required in the production of virulent virus should be autoclaved. Similarly, used embryos should also be autoclaved prior to disposal.

It is strongly recommended that the seed virus be prepared and stored in the production laboratory and then submitted to the quality control laboratory for approval. Full records should be kept of the origin, passage and storage of all seed virus (see Appendix 5). These records should include a statement of the type of eggs used for passage of the virus, the dates, and dilution levels of viral inocula. The use of seed virus for production lots should also be recorded. No batch of seed virus should be completely exhausted, because if later passage material is found unsuitable it will be necessary to revert to an earlier passage level. Wherever possible, seed virus should be stored both as freeze-dried material and as liquid virus frozen at —60°C. Hence in the event of damage to one container, an alternative supply of virus will be available.

Security of experimental animal facilities

These rooms will normally be used for both potency and challenge work in susceptible chickens, and must be located outside the production area. Where virulent virus is being used, the rooms must be provided with biologically filtered air exhaust systems equipped with roughing and absolute filters to prevent the escape of virulent virus. Provision must also be made for the effective sterilization of effluent water and of used litter and droppings. The latter should be incinerated in a burner located well away from the production laboratory. The containers used for the transfer of this material to the incinerator must be sterilized before re-use and should not come in contact with the production or diagnostic areas. Staff attending experimental animals must not be employed within the production area. This staff should be provided with protective clothing kept in the experimental unit. No staff engaged in other duties involving poultry should be allowed within the experimental animal facilities. It is necessary to design a system for transferring written records from the infected bird unit to the records office without this procedure becoming a source of contamination.

Where totally enclosed air-filtered units cannot be provided for the experimental animal facilities, a possible alternative is to use pens well removed from the laboratory and from all domestic poultry. These pens should be surrounded by bird-proof netting to prevent the spread of pathogens by free-flying birds. Experimental animal rooms and equipment may become contaminated from the regular use of vaccine virus undergoing test, and this virus may spread to experimental birds. To prevent this, thorough disinfection at intervals is very important.

SPF criteria for testing the source flock

It is essential that the production of the master and working seed material, and the passage techniques involved, be carried out with materials exposed to the minimum of contamination. With egg-propagated vaccines, these techniques should be based on SPF eggs. These are obtained from breeding flocks maintained in strict isolation and regularly and thoroughly tested for absence of poultry pathogens. It is highly recommended that SPF eggs be used for the final production of all live Newcastle disease vaccines.

The designation " specific pathogen-free " varies between countries. A list of the diseases for which tests are usually made, together with the type of tests employed, is given in Appendix 6.

Specific pathogen-free flocks may be developed at an appreciable dis-

tance from the vaccine laboratory, or SPF eggs may be purchased from companies or institutions specializing in this work. It should be recognized that many poultry pathogens are extremely ubiquitous, and unless strict isolation can be maintained, a disease outbreak may occur in an SPF flock (Cooper, 1970). If possible, birds should be housed in rooms supplied with filtered air under positive pressure (Drury *et al.*, 1969).

The degree of pathogen control in the production of eggs will vary from country to country and must be governed by local needs. However, it is essential that the seed viruses be grown and passaged in fully SPF material.

Isolator systems for SPF birds

An isolator is basically a sealed system in which birds are kept and for which there are safeguards to prevent contamination from air, feed, water, handling or cleaning. Air purity is usually obtained by absolute air filtration. Water direct from the main supply may be used because it can be assumed to be free of poultry pathogens, although not necessarily sterile; water can also be filtered as it enters the isolator.

Water may be metered into the plastic isolator by means of a reservoir which holds only a day's supply and is refilled from the main supply through a manually operated valve. This system has been found necessary because automatic drinker valves may fail to close, with serious results. The use of a reservoir allows the supervisor to record the amount of water used.

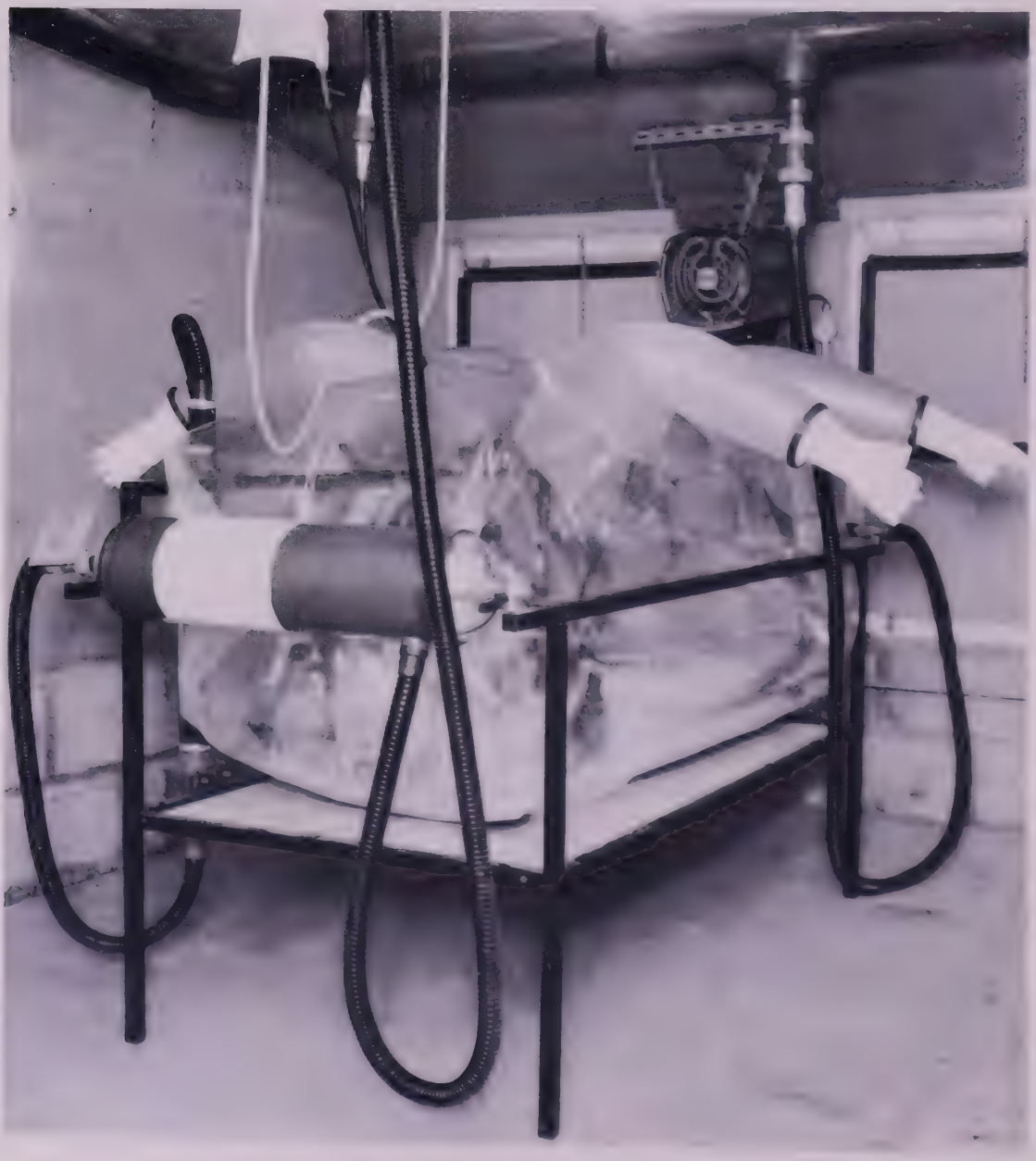
Feed may be sterilized by methyl bromide, ethylene oxide or some other chemical system, or it may be heat-treated (when extra vitamins will have to be supplied) or introduced in sealed bags direct from the feed mill. It may be convenient to have feed placed in double bags so that the outer bag can be immersed in disinfectant and then removed prior to using the feed from the inner bag.

Handling the birds is best done by the use of a sealed-in glove system so that the operator and the test poultry are never in direct contact. The isolator has two sets of gloved sleeves welded into the plastic. These sleeves have household-grade rubber gloves attached at the ends by means of a metal ring so that used gloves can be replaced when an isolator is being cleaned. Methods may be used whereby the ports are opened and staff operate with hands cleaned to surgical standards or with disposable gloves. This method of manipulation has been found to cause sterility breakdowns during an extended period of use.

Figure 3 shows a plastic bag which is the basis of an isolator system; the flexibility of the bag gives easier control of air flow. The advantages are low cost, ease of manufacture and good vision; the disadvantages are that

the bag may become torn, especially if large birds are housed in it; and in the event of an air failure, it will collapse (unless internally supported) and the birds will asphyxiate in a few minutes. To ensure a constant air supply, emergency air pumps are required as a safety measure in the event of a mains power failure or the failure of a single blower motor. This is the most expensive part of the development of isolators, and a triple system is advocated. The isolators can be driven by a vaned pump supplying 1.41 m^3 per minute at 25.4 cm water pressure. This pump is connected to another

FIGURE 3. Example of a flexible bag isolator



identical one by a series of microswitches, so that in the event of a motor failure the second pump cuts in. In addition, a third pump is connected to the system. This pump has the same output, but is driven by a 12-volt DC motor powered by a bank of automobile batteries. It cuts in automatically in the event of a power failure in the public electricity supply. Both standby pumps are connected to an alarm system so that whenever a malfunction occurs, an engineer may be called. One air supply system for a whole bank of isolators is preferable to individual motors for each isolator.

Air is ducted to the isolators by means of high-grade polyvinyl chloride (PVC) piping that can be cut, glued or bolted together by non-engineering staff, and the final connection between the isolator and the general ducting is made by means of flexible piping. Each isolator is supplied with air filters for incoming and outgoing air. Dust from chickens, composed mainly of feather debris, is generated at about the rate of 3 mg dust per kilo chicken weight per day. Absolute filters of the folded paper type may cost approximately U.S.$100 each to replace. A more economical system may be constructed from stainless steel filter frames made of welded wire. An ultrafine fibreglass roll is wound three times onto these frames. This fibreglass is taped in place with plastic tape and examined for presence of leaks. For incoming air that is basically very clean, the filter frame is sleeved into its outer frame. For outgoing air carrying the heavy dust load, muslin is wound on top of the glass fibre to trap coarser pieces of dust.

The filter and filter frames are taped inside an outer container and autoclaved. An ethylene oxide autoclave which operates at 60°C permits the use of plastics which can be sterilized; thus the outer filter frames can be made of PVC. In laboratories where this sterilization cannot be done, the filter frames must be made of metal.

Both input and output filters are clipped on to the outside of the isolator frame and are connected to the isolator and to the air supply systems by valved flexible tubing in a manner which permits a filter to be replaced by closing the valve. Air flow rates are checked by means of floating cone air flow monitors to ensure that the buildup of material on a dirty filter does not reduce the air supply to undesirable levels. These air flow monitors can be equipped with a photoelectric eye across the barrel to detect changes in the air flow rate.

Feed, syringes, bottles, and birds are placed in or removed from the isolator by means of a port which is situated between one pair of glove ports. This centre port is 30.48 cm in diameter and is a plastic tube covered at both ends with tight-fitting flexible plastic. The outer cover has two corked 1.27-cm outlets.

To place food in the isolator, a sealed bag is inserted into the port following the removal of the outer plastic cover. The cover is replaced and

one of the tubes is uncorked to allow 5 ml of 2 percent fresh peracetic acid to be sprayed into the port by use of a non-metal spray. The tube is corked and after 10 minutes the inner drum skin is removed by the operator by means of the glove system and the feed drawn into the inside of the isolator.

The isolator is welded shut at both ends after the essential containers such as the feeder and water drinker have been placed inside; alternatively, one end may be welded and the other clamped shut with a mastic compound which stays soft and allows the airtight seal to be broken at the end of the experiment. When the plastic isolator is sealed with mastic, it is then firmly clamped shut by means of a heavy-duty angle iron and bolts.

Excluding the cost of the air pump machinery, isolators of this type, with a floor space of 2 m^2, can be built for $200 each and can be operated at a cost of about $1 a week, including filter replacements.

Heavy-duty stainless steel isolators which have the same glove system for manipulations benefit from the replaceable air filter material, and can be operated with a porting system in which all contents are immersed in a disinfectant in a U-bend on the way to or out of the isolator. Lids must be kept in place to prevent the poultry from falling into the tank.

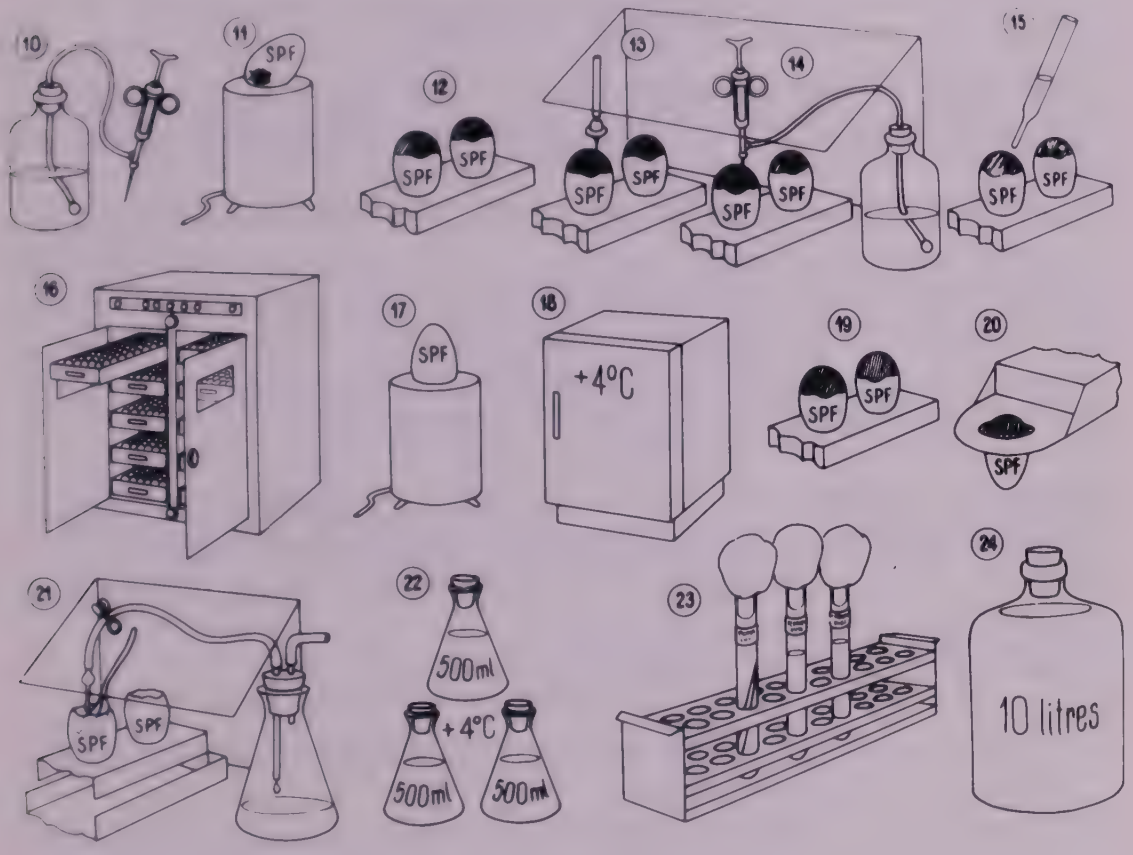
For producing SPF birds, the Houghton Poultry Research Station in the United Kingdom has developed an extremely efficient isolator made from fibreglass (Cooper, 1970). This isolator is not fully airtight, but works on the principle of a continuous stream of filtered air under slight positive pressure. Although expensive to make, it is one of the most effective means of keeping SPF stock.

Cooper and Timms (1972) have described a system which allows the rearing of chicks to maturity and their maintenance during the laying period under highly protected but not " germ-free " conditions. The isolator was 91 cm wide, 274 cm long and 74 cm from wire floor to roof. It accommodated 12 hens and one cockerel on a floor area of 2.2 m^2. The basic material used for the isolator was coated fibreglass which was resistant to sterilizing agents. Each isolator had its own ventilation unit comprising two fans, one being in reserve in case of a failure in the main fan. Prefilters and final air filters were fitted and a positive pressure of 4 cm water pressure was maintained. This type of isolator provided a method of rearing and maintaining day-old to 80-week-old chickens free from known viruses, bacteria and coccidia.

The establishment of a disease-free flock has been discussed by Hitchner (1975). The eggs should be hatched in an isolated incubator in a facility without other chickens. Rearing in environmentally controlled windowless houses with a filtered air positive-pressure ventilation system is preferred (Drury *et al.*, 1969). However, conventional poultry houses may be satisfactory if they are isolated and all openings are screened

against wild birds and rodents. Management procedures are important. Hence employees must not work with other poultry or keep poultry on their home premises. They must have a room where they can change, shower and dress in clean clothing and boots before entering the poultry

FIGURE 4. Flow diagram of the production of Newcastle disease vaccine
B. Vaccine production



Key to Figure 4

10. Preparation of virus dilution for egg inoculation
11. Candling and marking of the embryos
12. Disinfection of the egg shell on the air sac area
13. } Inoculation of the eggs with an automatic syringe under a hood
14. }
15. Closing of the eggs with liquid paraffin
16. Incubation of the virus-inoculated embryos
17. Candling of the embryos
18. Cooling of the embryos at +4°C overnight
19. Drilling of the egg shell on the air sac area
20. Cauterizing
21. Harvesting of the AAF under sterile conditions
22. AAF is harvested in smaller bottles, stored overnight at +4°C
23. Rapid testing for microbial contamination of the harvested AAF
24. Pooling of non-contaminated small bottles

house. All equipment and supplies introduced into the house should be disinfected, autoclaved or fumigated with formaldehyde. Disease surveillance should include post-mortem examination of all dead birds, and antibody tests as described in Appendix 6. Hitchner (1975) also indicated that blood samples for antibody tests should be obtained from every bird in the flock at 16 to 20 weeks of age.

Laboratory facilities

Laboratory facilities are used for two separate purposes: the production of a batch of vaccine, and the development or propagation of the vaccine seed virus.

It is essential that the production of the master seed material, and the passaging techniques which precede it, be carried out with materials exposed to the least possible contamination. With egg-propagated vaccines, these techniques should be based on the use of SPF eggs. These are obtained from breeding flocks maintained in strict isolation and regularly and thoroughly tested for absence of poultry pathogens.

Propagation facilities. The value of using SPF eggs and chickens is largely nullified if laboratory conditions allow the possibility of contamination. Hence the design and location of the laboratory must be adequate to prevent possible cross-contamination.

Basic requirements for a vaccine laboratory are:

1. Total separation from any area involving diagnostic procedures and the handling of pathogens.
2. Segregation of staff should be arranged so that personnel engaged in vaccine production will not come in contact with staff engaged in handling diseased or clinically healthy birds. No staff engaged in other duties involving poultry should be allowed access to the vaccine laboratory.
3. The staff working in the vaccine unit must change into sterile protective clothing in an antechamber before proceeding into the laboratory.
4. Materials entering the laboratory should be free from contamination and, if necessary, should be sterilized or treated with a disinfectant. It is important that the SPF eggs be brought into the working area with the minimum of outside contact.
5. Service engineers and other personnel having occasional duties inside the laboratory should follow the same rules of wearing protective clothing as adopted by the regular staff.

6. No person should enter the laboratory after immediate contact with healthy or diseased poultry or their products.
7. The laboratory should have a filtered air supply, under positive pressure, so that air leakage will be to the outside. Where facilities for a controlled atmosphere are not available, important work should always be carried out in a safety cabinet having a supply of filtered air. This cabinet should be situated in a clean room and should be operated by staff who follow the established rules and techniques.
   A safety cabinet consists of a work area of at least 0.5 m^2 enclosed by metal, but with a glass front panel. The cabinet is supplied with filtered air. The operator manipulates the materials by means of long plastic gloves fitted to ports in front of the cabinet. The cabinet should be capable of being sterilized by ultraviolet irradiation or by fumigation with formalin. For this purpose, laminar flow filters could also be used.
8. Eggs containing seed virus material should be incubated separately, if possible in a bench incubator which can be specially sterilized with a mixture of equal parts boiling water and formalin.
9. The harvesting of allantoic fluid containing seed virus should also be carried out under conditions of complete sterility, and containers of seed virus should be placed in refrigerators which do not contain contaminating material. Seed virus containers should be permanently labelled and a full record kept of the production process and subsequent storage.
10. Seed virus held prior to vaccine production should be subjected to the full range of safety tests as described in Chapter 7.

5. PROPAGATION OF VACCINE VIRUS

Inoculation technique

To produce amnio-allantoic fluid (AAF) that is completely bacteriologically sterile or which has minimal bacterial counts, the bacterial population in the work areas must be controlled as effectively as possible. One of the greatest dangers is from the eggs themselves, and care should be taken to fumigate them effectively before their introduction into the inoculation room. This will reduce the amount of shellborne contamination.

Egg shell surfaces are generally contaminated with a variety of microorganisms; thus after continual use the egg incubators also become contaminated. To reduce the microbial contamination, the incubator should be fumigated at regular intervals with formaldehyde gas.

Similarly, eggs may be effectively fumigated with formaldehyde gas in the incubator. Eggs should not be fumigated with formaldehyde between 24 and 100 hours after commencement of incubation because of the sensitivity of the embryo to formaldehyde at this age. Thus fumigation can be conducted within a few hours of the beginning of incubation. It is important to fumigate at 90 percent relative humidity, 37°C temperature, and with the fans running during the fumigation time. For 1 m^3 incubator space, 13 ml formalin (40 percent formaldehyde solution) and 6.5 g potassium permanganate are mixed in an enamelware vessel placed on the floor of the incubator. The maximum fumigation time is 20 minutes, after which the doors may be opened if necessary.

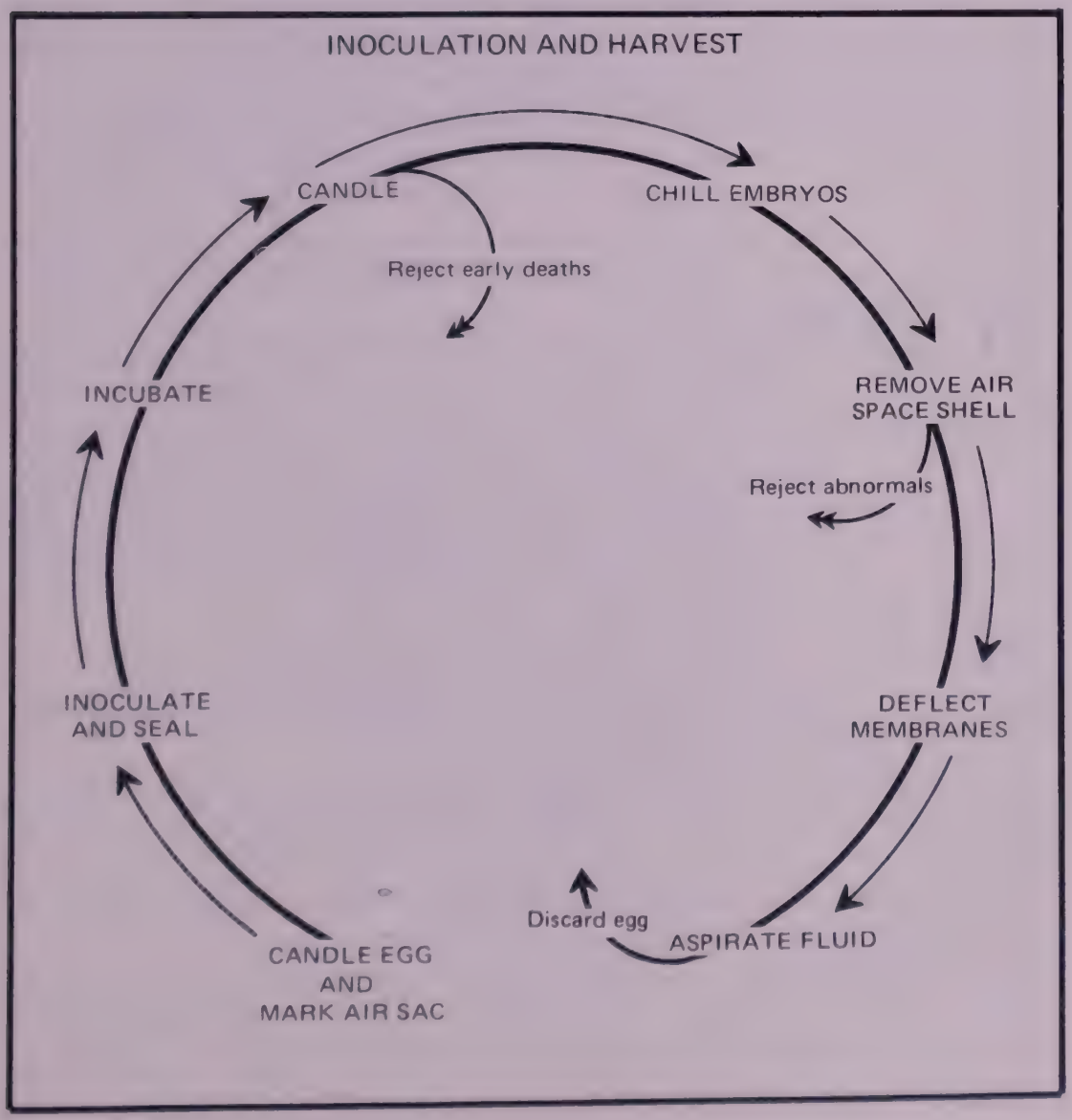
Embryonated eggs should be obtained from an SPF flock and transported to the vaccine production area under strict hygienic conditions. The optimal age for egg inoculation is 9 days' incubation. However, in the case of mesogenic strains of virus, 10- or 11-day embryonated eggs may produce an equally satisfactory harvest. Eggs of 8 days' or less embryonation tend to be more susceptible to non-specific death and provide a harvest of allantoic fluid which is low in virus titre.

Eggs should be candled before inoculation and all non-viable eggs removed. One of the main disadvantages of SPF eggs may be the relatively low viability of the embryos.

Inoculation of the eggs should be carried out under conditions of complete asepsis. The air sac area should be painted with a 70 percent alcohol distilled water mixture, tincture of iodine, or some other effective chemical sterilizing agent. The sterilizing agent should be allowed to dry thoroughly to prevent inactivation of the seed virus during inoculation.

It is also important that eggs be carefully candled before inoculation to ensure that all non-viable embryos are rejected. A badly contaminated embryo which reaches the harvest line may cause a high degree of contamination of the collected fluids. Staff should therefore be trained to reject all embryos that have an abnormal appearance.

FIGURE 5. Suggested scheme for the production of Newcastle disease vaccine



The following inoculation technique is recommended:

The margin of the air sac is marked in pencil with the aid of a candling lamp. This mark need not be more than 1 cm long and need not be related to the absence of blood vessels in the immediate area.

A hole is drilled or punched about 4 mm above the air sac margin. The hole should be approximately 1 mm in diameter to allow easy access of the inoculating needle, but sufficiently small to permit full and effective sealing after inoculation. Failure to seal the holes correctly may lead to contamination of the embryos with subsequent risk to the entire batch of vaccine.

Before the holes are made in the eggs, seed virus in excess of the quantities required should be prepared. A 10^{-5} dilution of seed virus material having an activity of at least $10^{9.5}EID_{50}$ per ml (Chapter 7) is suitable. Dilutions in excess of this may give rise to a small number of non-infected embryos, while lower dilutions may produce progeny virus of inferior quality. Dilution should be carried out in a suitable sterile diluent containing antibiotics. Sterile physiological saline, buffered to pH 7.0 with phosphate buffer and containing 200 units penicillin and 200 micrograms streptomycin per ml, is effective. Alternative diluents are tryptose broth or 10 percent horse serum. It should not be necessary to increase the levels of antibiotics. Bacterial contamination should be regarded as indicative of faulty technique, and should not be corrected by increasing the concentrations of antibiotics.

The seed virus suspension at a dilution of 10^{-5} may be inoculated into eggs using either a 1-ml tuberculin syringe or a repeating syringe drawing from a reservoir. The latter will help reduce contamination due to excess handling of the inoculating syringe. Sterility tests of the seed virus should be made from the inoculating syringe at the beginning and end of the inoculation work. The optimal volume for inoculation is 0.1 ml, although eggs can be inoculated with 0.2 ml. The needle used should be fine-bored. A suitable size is 0.5 mm × 11 mm.

When the inoculation is made with a 1-ml tuberculin syringe, it is advisable to change the needle and/or syringe after inoculating 120 embryos or the eggs on four egg trays.

The needle should be thrust down vertically through the hole in the shell (Figure 6) and the inoculum discharged into the lateral area of the allantoic cavity.

Embryo death from mechanical damage should not exceed 2 percent, and any increase above this level should be investigated.

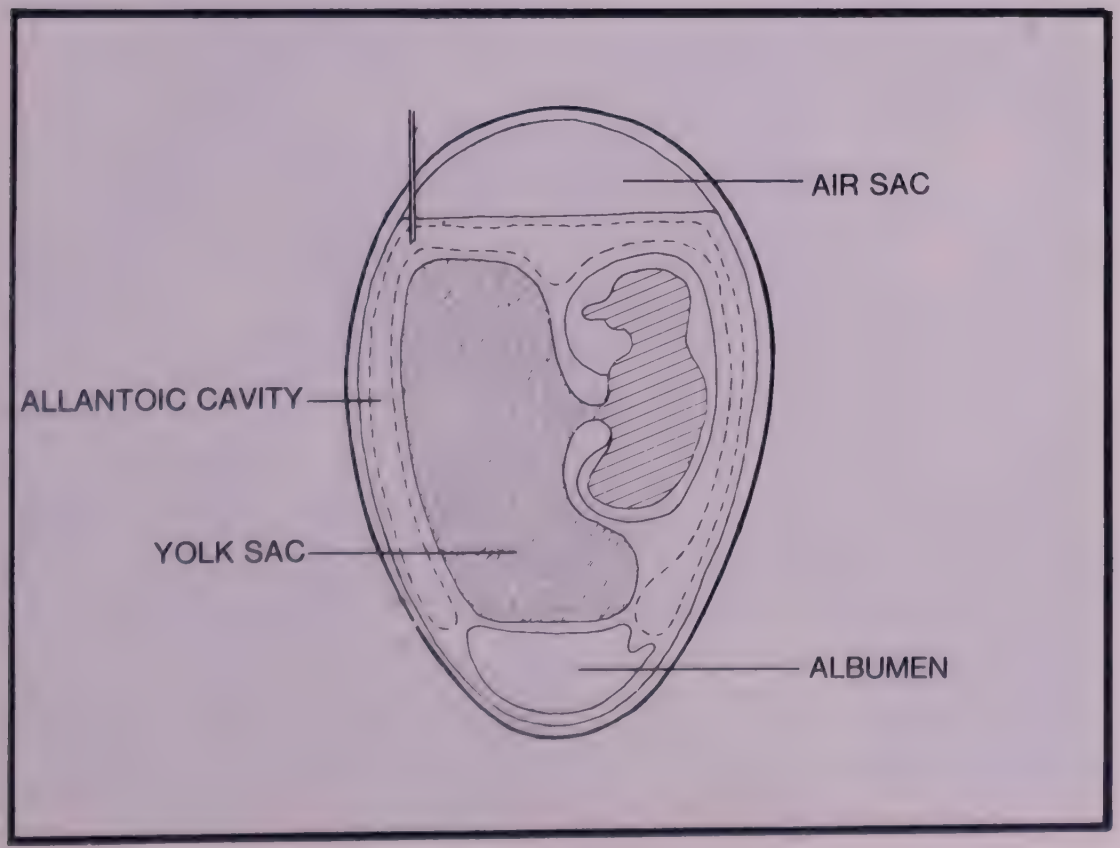
Care should be taken to avoid wetting the shell with excess inoculum, and all eggs should be sealed as soon after inoculation as possible. Several materials are available for sealing: the most popular are melted paraffin or a mixture of petroleum jelly and wax, or a coloured flexible collodion. The

collodion should be used away from a naked flame because the ether base is inflammable.

Following inoculation, the eggs should be incubated at 37°C in a well-ventilated incubator. Overloading a non-ventilated incubator may produce excessive levels of CO_2. The eggs should be candled within 24 hours of inoculation and all dead embryos discarded. The rejection rate should be under 2 percent. Poor viability of the embryos or excessive bacterial contamination are possible causes of non-specific early deaths. In some cases, mechanical damage at the time of inoculation may cause excessive mortality. Specific deaths due to virus infection do not occur during the first 24 hours after inoculation.

The batch volume depends on the capacity for processing the bulk harvest. This in turn is mainly dependent on the freeze-drying capacity. Inoculation of 2 000 eggs will yield between 6 and 12 litres allantoic fluid which will provide 6 to 12 million doses of vaccine. In many laboratories this batch volume has been found convenient. Smaller batches increase the testing costs per dose of vaccine, while larger batches require increased staff, and if continued in a routine manner may provide vaccine in excess of the needs of the country.

FIGURE 6. Allantoic cavity method of inoculation



Harvest technique

The harvest of infected amnio-allantoic fluids should be conducted when the virus infectivity titre is at its highest level. In the case of mesogenic vaccines, this titre occurs at, or soon after, embryonic death. Because dead embryos remaining in the incubator autolyse rapidly due to proteolytic enzymes and result in an inferior vaccine with low titre, care must be taken to remove dead embryos as soon as they are identified. Some laboratories harvest lentogenic strains at approximately 72 hours, when the majority of the embryos are still alive. It is not possible to define incubation periods precisely because these vary with the size of the egg, the temperature in various parts of the incubator, the strain and line of vaccine virus employed, and the dose of virus inoculum used.

The following incubation periods may be used as a guide:

F strain	96 hours
B1 strain	72 to 90 hours
La Sota strain	72 to 84 hours
Mesogenic strains	44 to 60 hours

The above incubation periods should be used as a guide only. The exact incubation time for each vaccine strain should be determined by the laboratory. The procedure recommended for mesogenic vaccine strains is to remove all the eggs from the incubator when about 90 percent of embryos have died.

The embryos removed from the incubator should be grouped as follows:

— Embryos dead after more than 24 hours' incubation, but before the mean death time (Chapter 9). These embryos should be discarded.
— Embryos dead at the time of removal from the incubator at the end of the incubation period.
— Embryos alive at the time of removal.

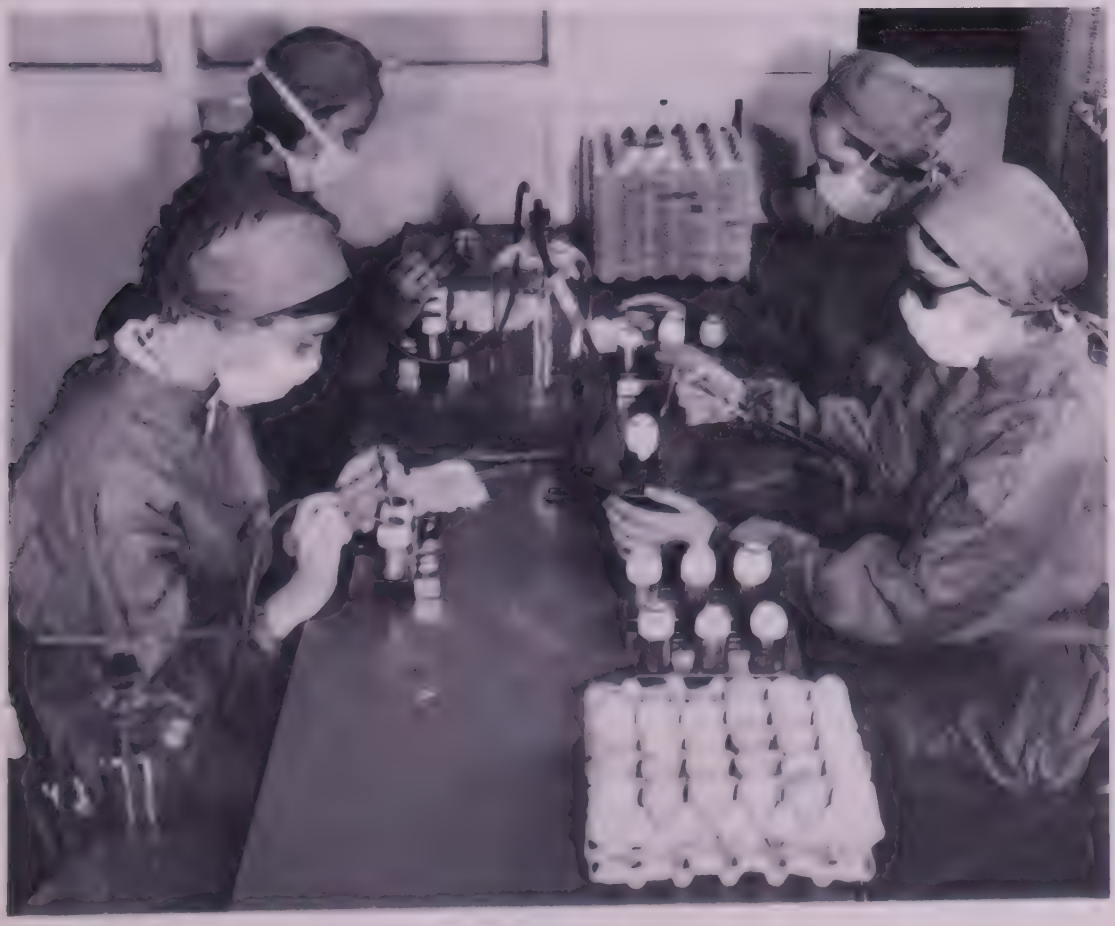
The following sequences of harvesting should be adhered to carefully. The embryos which died at the mean death time should be harvested first. Following this harvest, live embryos should be harvested after they have been chilled for at least 4 hours at +4°C to ensure that the blood vessels are empty before the membranes are reflected.

Generally, however, it will be found more practical to chill all the eggs overnight at +4°C before harvesting. The eggs should then be allowed to regain room temperature in a dry atmosphere in order to prevent excessive condensation on the shells which may cause contamination of the harvest

material. Following this, the top of each egg should be sterilized with a bactericidal agent, for example, 70 percent alcohol or 1 percent iodine in alcohol. The sterilization of egg surfaces with iodine solution is recommended. This is best conducted with a brush or cotton swab. Spraying of iodine can be dangerous to the operators. The sterilizing solution should be allowed to dry before the operator proceeds to remove the egg shell above the air space. This latter procedure may be carried out with forceps, diamond-pointed cutting wheels, hot cautery wires, a small Bunsen flame, or with a circular drill combined with a suction device which will cut off the top of the egg shell and remove the cutting dust by negative pressure. Figure 7 illustrates harvesting in progress.

A relatively small number of eggs should have the shells removed at one time. This operation and those following should be carried out under a sterile hood. The egg membranes should be reflected from both the allantoic and amniotic cavities, using a pair of fine-pointed forceps. During the harvesting process, the operators should inspect each egg prior to aspiration of the allantoic fluid. Any egg visibly contaminated must be

FIGURE 7. Harvesting in progress



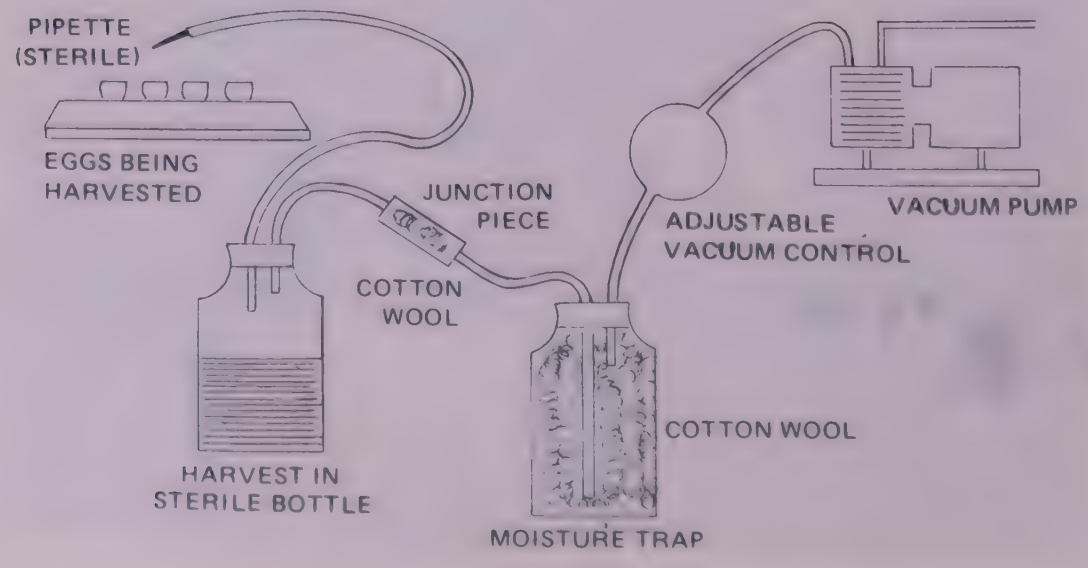
rejected. If contaminated eggs are inadvertently harvested, the high bacterial contamination level of the AAF may make the inactivation of virus difficult. Thus operators should recognize the cloudy appearance of the fluids of contaminated eggs. The operators must wear sterile gowns, caps and masks during the process.

Aspiration of the fluids is best carried out using a vacuum pump adjusted to a vacuum of approximately 12.5 cm of water, to which are attached the harvesting bottles, the pipeline and the glass pipette (Figure 8). Where possible, the pump itself should be located outside the harvest room to prevent the exhaust from the pump re-entering the working area. The pump can be connected to the room by a vacuum line, and switching facilities should be inside the room.

The fluids should be harvested into containers having a volume of 500 to 1 000 ml. Thus any contaminated container may be discarded with less loss. A supply of these sterile containers should be maintained double-wrapped, ready for connection to the harvesting system. Sterile plastic tubing and sterile aspirating pipettes should be available in sufficient numbers. These should be individually wrapped and changed every time a new harvesting bottle is used or a visibly contaminated egg is handled accidentally. The aspirating pipette should be blunted to prevent damage to the yolk sac membranes, and it may be convenient to locate the orifice slightly to one side of the tip to prevent its being closed by loose flaps of membrane.

Care should be taken to aspirate the amniotic as well as the allantoic

FIGURE 8. Harvesting of Newcastle disease vaccine



fluid. If the amniotic sac is not opened by the spatula or another instrument, the sac may be pricked by the aspirating pipette. The yolk sac must not be damaged, and a container of vaccine with excessive yolk should be discarded.

To assist in the efficient aspiration of as much fluid as possible and allow better penetration of the cavity, a sterile spatula, small spoon or forceps should be used to depress the embryo and yolk sac. If the pipette is inserted too deeply, albumen may be aspirated. This should be avoided, although very small amounts of contaminating albumen may be disregarded.

As the harvest progresses, the bottles should be removed, closed, numbered and placed immediately in a refrigerator at +4°C. At the completion of the harvest, all bottles should be subjected to a simple bacteriological sterility test to detect gross contamination. Blood agar plates inoculated with 0.1 ml of harvested fluid and incubated aerobically and anaerobically will generally suffice.

Freeze-drying

Freeze-drying should be carried out within 24 hours of harvesting. Meanwhile, stored fluids should be held at +4°C and should not be frozen. Freezing and subsequent thawing will tend to lower the virus titre and damage the antigen.

All harvest bottles found to have less than 10 colonies per 0.1 ml on the blood agar plates should be pooled, and sterile skim milk powder or another suitable stabilizer added at the rate of 5 percent weight/volume. This must be mixed well, before the vaccine is dispensed into the vials. For a 1 000-dose vial, the liquid content may vary from 1 to 5 ml (Appendix 4). Vials or ampoules should be chemically clean, free from all traces of detergents, double-rinsed in distilled water and sterilized by dry heat at 160°C for 60 minutes. Trays of vials should be wrapped to maintain sterility until required. The vials should be filled under a sterile hood (Figure 9). Caps should be placed loosely on the vials in an aseptic manner, taking care not to seal the vials and thus prevent evaporation of the water during the freeze-drying process.

It is not possible to describe the precise details involved in freeze-drying, as these depend on the particular equipment in use. The procedure is shown schematically in Figure 10. The following points should be considered:

1. It is important that the vials be frozen to —40°C before a vacuum is applied, unless a centrifugal freeze-drier is used.
2. The freeze-drier should be well maintained. Contamination of the

vacuum pump, leakage or inefficient condenser operation will reduce the vacuum level and may result in a product that is not sufficiently dry. A drier that cannot produce less than 100 microns vacuum is of little value; a poor vacuum may even cause melting of the vaccine.

3. Heat to accelerate the drying process should be applied carefully to ensure that the temperature does not rise above the eutectic point. During the secondary drying stage the shelf temperature will usually have to be raised above 0°C to complete the process in a relatively short time.
4. Vials should be crimped (sealed with a metal collar) as soon as possible after stoppering in the chamber and subsequent removal from the freeze-drier. If vacuum-sealed, all vials should be tested before storage with a high-frequency tester for the presence of luminescence, which shows a sufficiently high vacuum. Vials which fail to fluoresce should be discarded, and the percentage recorded. When vials are filled with an inert gas such as nitrogen, testing in this way is not possible.

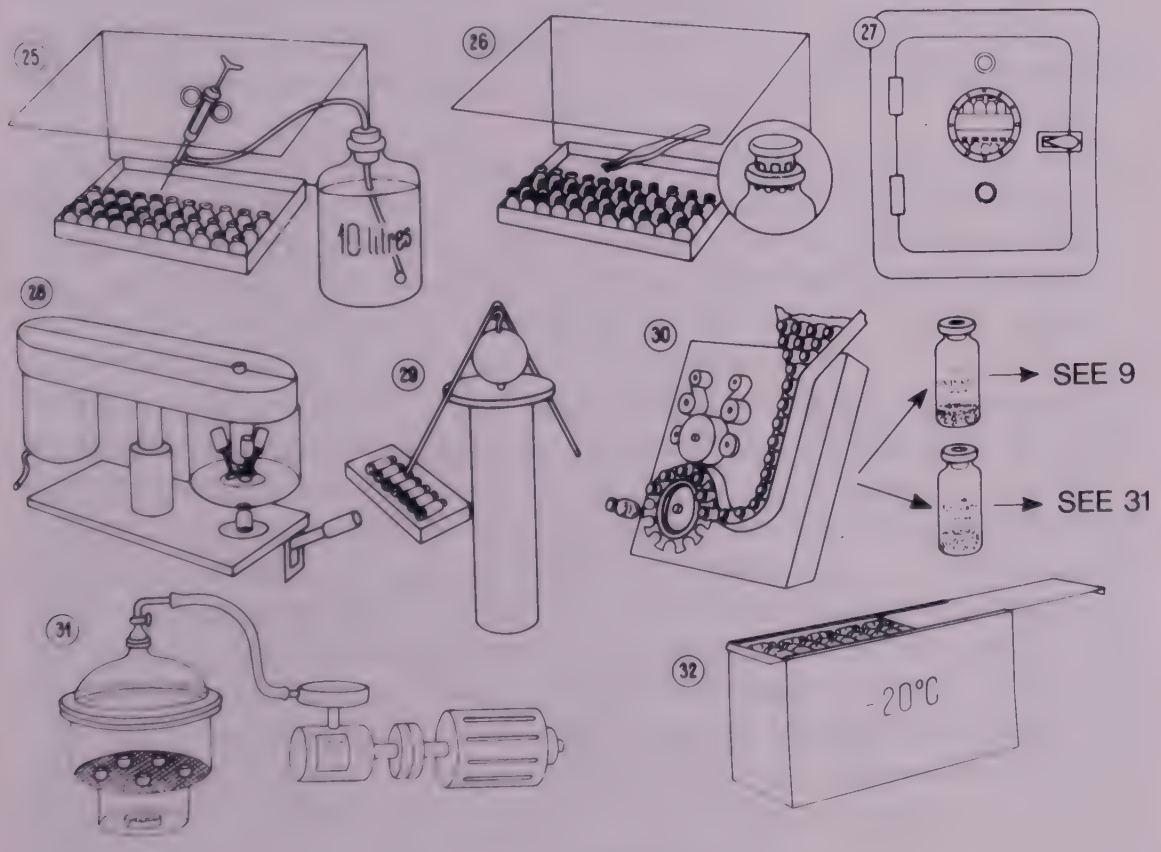
FIGURE 9. Vial filling in progress

5. After testing for vacuum, the vials should be marked and placed in aluminium boxes for storage.

Storage and transport of vaccine

Freeze-dried vaccine should be kept at an even temperature, preferably at —20°C. Storage at room temperature is not recommended. During storage, care should be taken not to allow any marked temperature fluctuations to occur.

FIGURE 10. Flow diagram of the production of Newcastle disease vaccine
C. Freeze-drying



Key to Figure 10

25. Filling of vaccine under sterile conditions
26. Stoppering of vials
27. Freeze-drying
28. Fastening stoppers with a metal collar
29. Testing for vacuum with high-frequency equipment
30. Marking of vials and sampling for potency and residual moisture tests
31. Determination of residual moisture
32. Storage of the finished vaccine at —20°C

Transport from the manufacturing laboratory to distribution points should be as rapid as possible. Well-packaged vials can be expected to maintain a temperature below 0°C for at least 24 hours, provided they are not exposed to direct sunlight. Transport in unventilated vans must be avoided because the interior temperature may become excessively high. Vehicles should go directly from the point of collection to their destination without long stops en route. At distribution points the vaccine should again be stored at —20°C. Users of the vaccine who do not have adequate storage facilities should be encouraged to draw from the distribution point. Vaccine should not be held for more than a few days at 0°C.

As the viability of the vaccine will depend partly on the efficiency of the freeze-drying, it is recommended that manufacturers carry out stability tests on their products as follows:

1. Potency test at the time of issue (Chapter 7).
2. Potency tests after storage for six months and one year by the manufacturer.
3. Potency test following transport to a farm, storage on the farm for 14 days, and return to the manufacturer's laboratory. This involves a test in susceptible chickens. It is not sufficient to conduct virus titrations because a high infectivity titre does not necessarily indicate full potency.

When freeze-drying is not undertaken and the vaccine is used directly from the undried frozen state, storage and transport present considerable problems. Undried frozen virus should be stored at —70°C, as higher temperatures will cause decay in the viability of the vaccine. Balla (1960) found that Newcastle disease H and B1 vaccines stored in the undried frozen state at —35°C retained their original titres for eight months. After thawing and keeping for seven days at +25°C, an average drop of 0.7 log occurred. If these vaccines were stored at —20°C or —10°C, a drop of 1.4 and 3.5 log respectively was observed after an eight-month storage followed by seven days at +25°C. Transport of the vaccine should be effected with adequate supplies of dry ice (solid CO_2) in well-insulated boxes. The vaccine should be kept surrounded by dry ice until thawing prior to use.

Great care should be taken in the handling of insulated containers because they may become a vehicle for the transfer of field virus.

Undried frozen vaccine should be used as soon as possible after manufacture, and the storage period should never exceed four months at —35°C.

Undried vaccines may be dispatched with an expiration date not exceeding seven days when in vacuum flasks containing ice.

6. INACTIVATED VACCINES

Inactivated vaccines have been used since 1953 (Hofstad, 1953) and studies were published on the use of whole embryo vaccines by Grun and Hudson (1966). Later, Piercy *et al.* (1964) reported the use of aluminium hydroxide adsorbed vaccines prepared from infected allantoic fluids and vaccines of this type were in wide use in the 1960s. Nedelciu and Dinculescu (1965) examined the use of oil emulsion inactivated vaccines. This report was followed by a review of the use of oil vaccines (Zanella, 1970).

Extensive use of the oil emulsion vaccines during the epizootic in the United Kingdom in 1970-71 (Phillips, 1973) and in some countries of Europe (Zanella, 1966) has confirmed their value, first by serological demonstration of the extremely high levels of antibodies they produced, and later by the clinical demonstration of freedom of vaccinated birds from both overt disease and any drop in egg production. The success of a programme based on the use of live vaccines during the growing period, followed by one or more injections of oil emulsion inactivated vaccine, has now been confirmed (Phillips, 1973).

Only inactivated vaccines of high potency are able to give effective long-term immunity. The application of these vaccines can be part of the vaccination programme. Not all inactivated vaccines are of high quality and not all vaccination procedures are conducted at the correct interval after an effective primary dose of vaccine. When the primary dose was ineffective, the use of the inactivated vaccine resulted in only a primary response and the much greater value of a good secondary or tertiary response was lost.

Methods of production

Source of antigen. Inactivated vaccines are dependent on the antigenic mass for immunogenicity, and as the vaccines are non-living, no multiplication of antigen takes place within the body. Therefore it is essential that an adequate level of antigen be introduced into the tissues. Newcastle disease virus grows to a higher titre if lentogenic virus is used as seed material rather than velogenic virus. Studies (Gough *et al.*, 1977) have

indicated that the Ulster 2C strain is suitable because of the high EID_{50} growth potential.

Suitable strains for inactivated vaccine production include the F, B1, La Sota and Ulster 2C. Gough and Allan (1974) have indicated that the Ulster 2C strain is superior because of the relative stability of the antigenic material.

Choice of inactivating agent. Betapropiolactone (BPL) is now more commonly used as an inactivating agent than formalin, although the latter can result in a good-quality vaccine. BPL has the advantage of acting very rapidly, and although the chemical itself is regarded as having a carcinogenic hazard, it is degraded to propionic acid within a few minutes of coming in contact with organic material and so becomes non-toxic. Another benefit from the choice of BPL is that it is able to inactivate the leucosis agent and hence allow inactivated vaccines to be made from non-leucosis-tested eggs, an aspect that might not be completely safe if formalin is used as the inactivating agent.

Experience with some aluminium hydroxide adsorbed inactivated vaccines has suggested that use of formalin as an inactivating agent may result in a more stable vaccine than would use of BPL (Allan, 1963, unpublished). This has been substantiated by Garlick and Avery (1976).

Inactivation of the virus. Eggs used for the manufacture of inactivated vaccine should be produced by flocks which are fully susceptible to Newcastle disease. Large eggs should be used in preference to pullet eggs, because the latter can result in lower yields of virus. The inoculation of eggs, chilling of embryos and harvesting are as described for live virus vaccines. Inactivated vaccines must be bacteriologically sterile, unlike many live virus vaccines in which a limited number of non-pathogenic organisms may be permissible.

The harvested fluids should be aspirated into a chilled container to prevent bacterial multiplication prior to inactivation.

Inactivation may be achieved with formalin at a final concentration of 1:1 000. The formalin used should be of laboratory grade, and bottles in which there are traces of polymer precipitate (paraformaldehyde) should not be used. It is advisable to add a 1:10 solution of formalin to the bulk container using aseptic techniques. The contents must be well stirred during the addition of the formalin. After adding the required formalin, the fluids should be transferred to a second container to ensure that any virus fluids splashed on the neck of the vessel do not escape the inactivation process. Inactivation should be continued for 16 hours at 37°C and allowance made for the time required for the bulk container to reach incubation temperature.

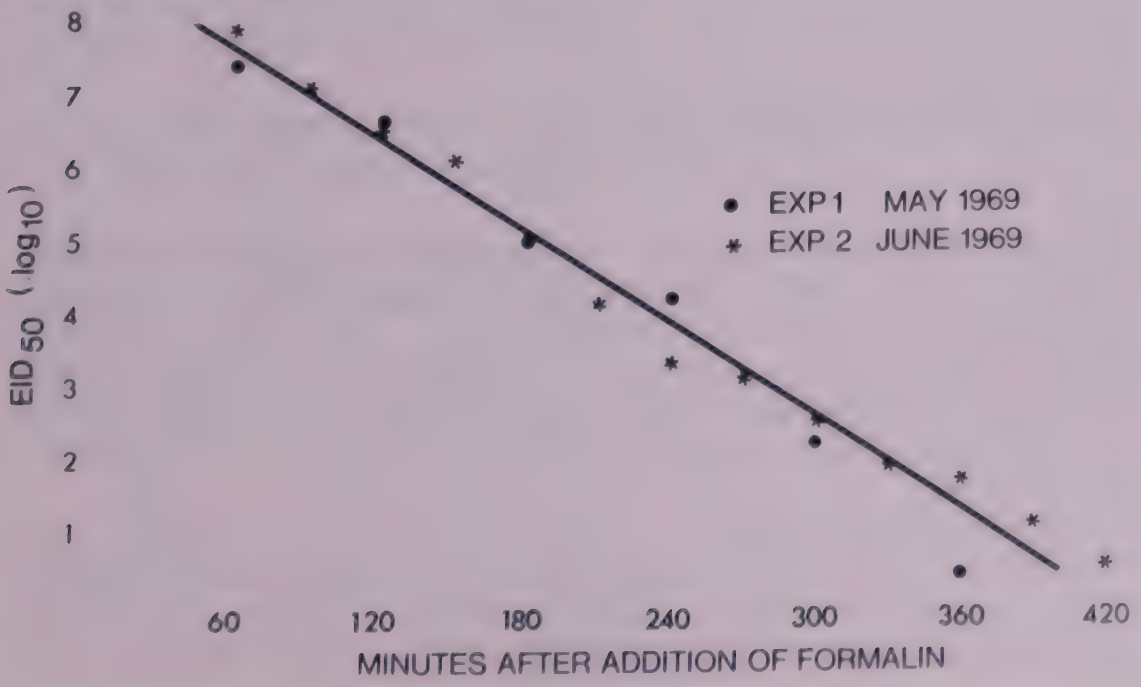
Figure 11 shows the kinetics of the inactivation of Newcastle disease virus by formalin. The inactivation process follows first-order kinetics and is log-linear. The figure can be used to predict the probability of infective virus remaining after a given time at 37°C. The log concentration of Newcastle disease virus drops by a factor of 1:10 at approximately 50-minute intervals. Thus, with an initial titre of 10^8 infective particles per 0.1 ml, 470 minutes after the beginning of inactivation there is less than 0.1 particle, and 520 minutes after the start there is less than 0.01 particle in 0.1 ml. Inactivation overnight during a 16-hour period will give a probability of 60 ^{8} infective particles per 0.1 ml of fluid, and thus can be taken as a reasonable level for full inactivation.

Following inactivation, the sterile bulk container should be stored at +4°C while tests for bacterial, viral and fungal contaminants are conducted (Chapter 7). Should the product be contaminated, reinactivation will be required, and this will cause a reduction in immunogenicity of the vaccine.

BPL at a final concentration of 1:2 000 may be used for inactivation. Inactivation should be conducted as described above, except that the reaction time with BPL may be reduced to 90 minutes at 37°C followed by rechilling at +4°C.

Vaccines prepared by inactivation using either of the preceding processes have been found to be potent. However, it has been observed that

FIGURE 11. Inactivation of the Ulster 2C strain with formaldehyde at 1:1 000 at 37°C (allantoic fluid pH 7.0)



BPL inactivation is more effective in destroying bacterial contaminants. BPL is unstable, and some difficulty may be found in maintaining the reagent at full activity. It should be stored at +4°C. There is no simple test for the activity of BPL.

Inactivation testing. Total viral inactivation should be demonstrated. This test should consist of sampling not less than 5 ml from each container of inactivated AAF and inoculating 0.2-ml aliquots into 25 nine-day embryonating eggs. The inoculated eggs should be candled twice daily for seven days. At the end of this period the eggs should be chilled and the AAF harvested from each egg and tested for haemagglutinin. The fluids of all eggs dying on test should be pooled and a second group of 25 eggs inoculated. A third group of 25 eggs are inoculated with the pooled fluids of those eggs of the second group remaining alive at the end of the seven-day period.

The second and third passage in eggs should be similarly examined for haemagglutinin. The inactivated vaccine can be considered free from Newcastle disease live virus if no haemagglutinin is detected in any of the three passage tests. The fluids should also be tested for bacterial sterility as described in Appendix 6. After completion of all the above tests the AAF may be emulsified with oil to form the final vaccine.

Immune response. Figure 12 illustrates the comparative effects of two formol-inactivated vaccines: one with a high antigen content derived from a lentogenic virus and the other with a lower antigenic content derived from a velogenic virus. The HI results show that the log mean primary response following the use of the lentogenic inactivated vaccine is considerably higher at the peak response three weeks after the initial vaccination. Following the second vaccination, the difference in response between the high- and low-titre vaccines is less.

The secondary response to inactivated vaccine is higher than the response from revaccination with live lentogenic vaccines (Figure 19).

Inactivated oil emulsion vaccines. The emulsion may be of the oil-in-water or the water-in-oil type, and may be a single emulsion or a double (oil-in-water-in-oil) emulsion. The oil selected for use may be a fine-grade mineral oil (e.g., liquid paraffin) or a vegetable oil (e.g., groundnut oil). Different amounts of antigen in the aqueous phase, oil and emulsifier can be used. The oil and the emulsifier should be sterilized by autoclaving prior to mixing.

In the formula reported by Gough *et al.*, (1977), the vaccines were emulsified as a 12.5 percent emulsion in an incomplete Freund's adjuvant which consisted of laboratory-grade liquid paraffin (86.25 percent) and the emulsifier Arlacel (1.25 percent).

Aluminium hydroxide gel, containing 2 percent $Al(OH)_3$ is commonly used as a mixture of equal volumes of aluminium hydroxide gel and inactivated AAF.

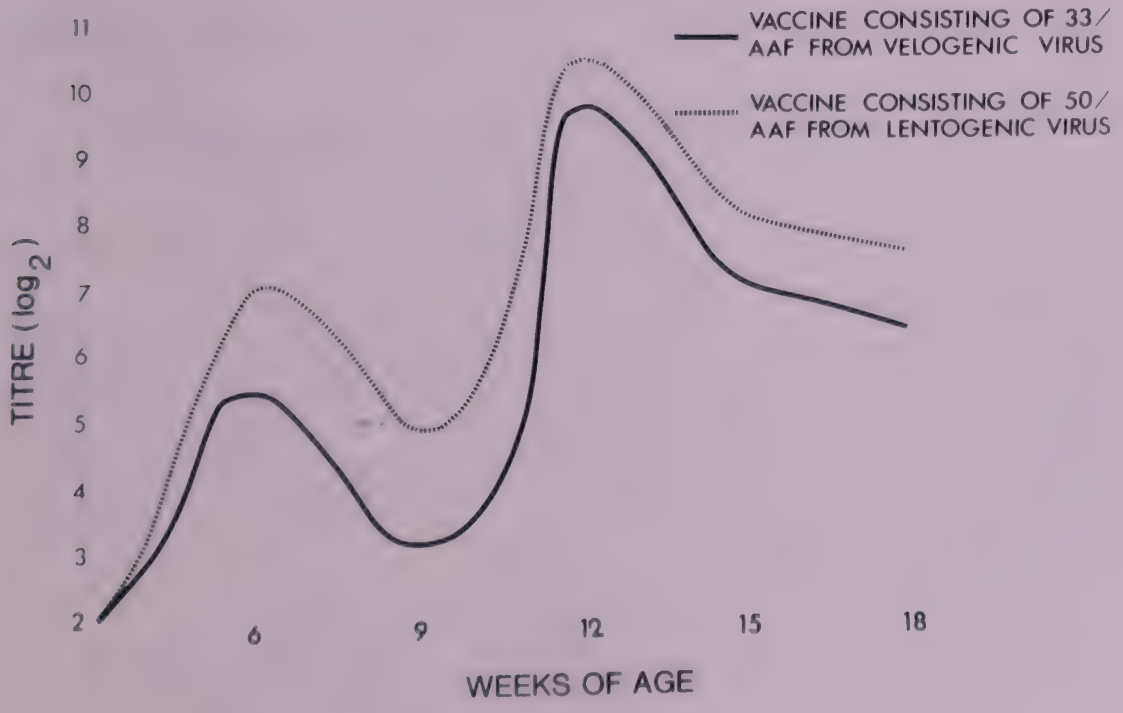
Stabilized emulsions of inactivated AAF in an oil base must be prepared with care to ensure a stable emulsion. One type of emulsion consists of 90 percent mineral oil (for example, Dracheol No. 6V) and 10 percent Arlacel A emulsifier. Four parts of the emulsion are added to one part of inactivated AAF and emulsified.

Another formula is groundnut oil 86 percent; aluminium stearate 4 percent and Arlacel A emulsifier 10 percent. In this vaccine, equal volumes of inactivated virus and emulsion are used.

For commercial quantities of vaccine, emulsifying machines such as the Silverson Blender or an ultrasonic emulsifier should be used to create the emulsion.

An aqueous phase of AAF significantly above 12.5 percent can produce emulsions that are too thick to be easily injected. If the antigen content is to be increased, either a different formulation must be used or a concentration technique employed. Examples of the latter are centrifugation with a continuous flow rotor, or dialysis and concentration using equipment similar to the Amicon hollow fibre ultra dialysis system. The latter system has been used to increase antigen concentration from the AAF without recourse to centrifugation.

FIGURE 12. HI response to formol-inactivated vaccines of different potencies



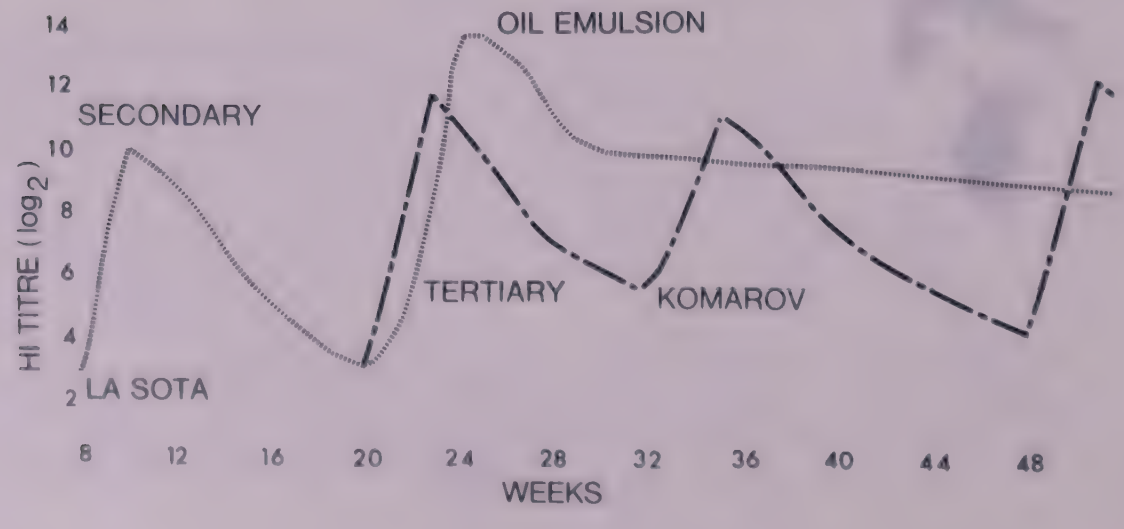
Sterility testing. Owing to the non-aqueous nature of the vaccine, sterility testing may be more of a problem than for aqueous products. It is suggested that a significant number of samples of the vaccine, both from the bulk container and from the final product bottles, be plated out on different selective media.

The contents of a bottle contaminated after part of the contents has been used may constitute a serious hazard when the balance of the vaccine is injected. All vaccine bottles should be accompanied by instructions indicating that they are to be used completely on the day of opening.

Injection routes and dosage. Vaccine may be given by either intramuscular or subcutaneous injection, but should not be used for birds destined for human consumption within four weeks after vaccination, because the carcasses might be downgraded for a residual swelling at the vaccination site. The degree of tissue reaction varies considerably with the brand of vaccine used. Some commercial vaccines cause little residual tissue reaction after one week, while other vaccines result in visible lesions lasting for over a month. Some veterinarians have suggested that the injection be given subcutaneously at the base of the head of the bird in order to use a site which would be discarded during the trimming of the carcass. Investigations of adverse reactions to Newcastle disease vaccination using oil vaccines have shown that unskilled vaccinators may seriously misplace the injection. Therefore, it is important that those conducting the vaccination ensure that the vaccine is administered with care and not at an excessive speed. In the event of a needle breaking during the injection process, the bird involved must be destroyed immediately.

The inner thigh muscle is a common site for the intramuscular injection

FIGURE 13. Comparison of oil emulsion and Komarov booster vaccination



of oil emulsion vaccines because the needle can be seen to be directly placed into the muscle.

The dosage for chickens and for turkeys below 10 weeks of age is 0.5 ml; for turkeys older than 10 weeks and for other larger birds the dose is 1.0 ml to 2 ml.

Figure 13 shows the immune response when an oil vaccine is correctly used as a booster dose. Experience has suggested that when a primary dose of live vaccine is followed by a secondary dose of inactivated oil vaccine, the antibody response is satisfactory. Thus, HI titres in excess of 2^{13} have been obtained as peak responses and the mean log HI titres for the flock may remain at or above 2^{10} for more than 20 weeks. Only under the most severe epizootic conditions has it been necessary to increase the immunity by a third vaccination at 40 to 45 weeks of age. Reports on the efficacy of oil emulsion vaccines include those by Allan (1972) and Box and Furminger (1975).

7. POTENCY AND OTHER TESTS

Different estimations of vaccine potency may be employed. These are:
— The EID_{50} content of a reconstituted vial of vaccine.
— The HI levels in a group of vaccinated chickens.
— The potency test in chickens by challenge.

Newcastle disease virus may be assayed either by estimating the haemagglutinin content or the infectivity of a sample of virus. It is recommended that the estimate of the haemagglutinin content be confined to serological work connected with the HI test, or to tests for the specificity of the viral preparations. The reason for this recommendation is that the haemagglutinin reaction estimates only the red cell agglutinating power of the sample. This reaction may be due to virus that is not active, or to viral subunits. In either case, the haemagglutinin reaction is not a reliable estimate of the amount of viable virus contained in a sample of vaccine.

For this reason, the most important control work relating to vaccine testing is based on a virus infectivity assay. For this assay to be significant, it must be carried out with precision. Undetected errors occurring in the assay system may lead to incorrect estimations of the quantity of virus present. To increase the accuracy, it is useful to carry out a series of replicate titrations to determine the reliability of the system being used. It is recommended that the calculation of virus end points be based on the Spearman Karber method (Finney, 1964) rather than the Reed and Meunch method. Accurate titration of the viral content is required to ensure that each vial contains the optimal concentration of virus, and to estimate the small loss of titre that occurs during freeze-drying and storage of the finished material.

The assay systems generally used are based on the inoculation of eggs or tissue cultures with tenfold dilutions of the virus. It is possible to have an estimate of Newcastle disease viral content before titration is commenced. This permits a narrower range of dilution levels to be titrated and adds considerably to the accuracy of the assay without involving additional work. Newcastle disease virus in infected allantoic fluid harvested at death of the embryo and titrated after storage at +4°C rather than after a cycle of freezing and thawing can be expected to have a titre of approximately $10^{9.0}$ EID_{50}/0.1 ml. Lentogenic virus of the B1 type usually grows to a slightly greater titre, and some samples of allantoic fluid may contain virus at $10^{9.4}$

EID_{50}/0.1 ml. For practical purposes, fresh allantoic fluid of velogenic virus strains may be assumed to have a concentration of $10^{9.0}$ EID_{50}. From these values, the titration assay may be conducted over a narrower range of dilutions.

Serological estimations of potency. The potency of a vaccine can be defined as its ability to protect a bird from disease. Laboratory assay systems and challenge tests with experimental birds are methods of estimating the potency of a vaccine. A potency test may be of the pass-or-fail type, or may provide an estimate of potency which will allow comparisons between various batches of the vaccine. The latter is considered more useful. With inactivated vaccines, it is possible to obtain an end point value by a challenge system based on inoculating increasing dilutions of the vaccine into groups of birds and calculating the 50 percent protective level. With live vaccines this procedure is less reliable, and challenge test systems have usually been designed to ensure that the vaccine meets certain minimal requirements, rather than to determine the accurate level of protection.

If the activity of the vaccine is estimated by the serological response in groups of experimental birds, then the potency may be measured without the use of virulent challenge virus (Chu and Rizk, 1971). This aspect may be very important where laboratory facilities do not exclude the possibility of contamination of the vaccine with virulent virus.

The embryo-infective dose 50 percent end point

Newcastle disease virus is relatively stable and simple diluents may be used. The cheapest diluent is sterile physiological saline produced by the addition of 8.5 grams of NaCl per litre of distilled water. The pH should be adjusted to between 7.2 and 7.4 by the addition of phosphate buffer. The saline should be autoclaved to ensure its sterility, and it is useful to add penicillin at 200 units per ml and streptomycin at 200 micrograms per ml final concentrations. These antibiotics will control many bacterial contaminants which may be present in infected fluids, and which could give rise to false death patterns in the inoculated eggs. Working with known sterile material under aseptic precautions allows the antibiotics to be omitted.

Dilutions may be made in capped tubes or in " universal " bottles. These must be sterilized before use and the sterile supplies must be stored away from the bench area where infective virus is being used. Pipettes should be of the grade B type and should be as uniform as possible; that is, they should be obtained from one supplier and should all have the same type of markings. The jet aperture should be such that the emptying time is not shorter than 20 seconds and not longer than 40 seconds for a 1-ml

pipette. Flow rates beyond these limits may cause errors or make the titrations inconveniently slow. Pipettes must be sterilized before use, either in canisters or individually wrapped. Disposable pipettes may be used provided they conform to the grade B specification.

Instead of using sterile pipettes, tuberculin syringes fitted with long hypodermic needles may be used. The use of syringes is less accurate, but if adopted, the dilution series should be made in capped 10-ml tubes.

In laboratories where it is not possible to produce a large quantity of sterile glassware for titration purposes, this modified technique using sterile tuberculin syringes may be of value. The syringes should be sterilized by heat and not by boiling, because the use of wet glassware will increase the error of the dilution steps to the point where the interpretation of the results will be of limited value.

For the tenfold dilution series, the capped tubes should be filled with 4.5 ml diluent and have 0.5 ml virus material added. For the fivefold dilution series, tubes should be filled with 4 ml diluent to which is added 1 ml of virus material.

Preparing a tenfold dilution series. Nine ml of diluent are added to each of 10 bottles using a 10-ml pipette. Each bottle should be marked from 10^{-1} to 10^{-10}.

One ml of the virus material to be assayed is transferred into the first bottle, using a 2-ml level down to the 1-ml level. The pipette should be held vertically and the level read from the bottom of the meniscus. The pipette should be filled by using a rubber teat to avoid contaminating the mouth of the operator. All pipettes should be plugged with cotton wool before sterilization. If the cotton wool is wetted accidentally during the titration, that pipette and teat should be discarded and new ones used in their place.

Control of the pipette during the delivery phase is best achieved by finger tip closure of the top of the pipette, and with practice it is possible to learn to overfill the pipette by means of the rubber teat, and then transfer control to the finger and adjust the contents to exactly 2 ml before starting delivery.

The pipette and the remaining 1 ml of virus material should be discarded after delivery. The contents of the bottle are mixed by using a new 2-ml pipette and teat, and filling and emptying ten times. Using the same pipette, 1 ml of the virus dilution (1:10) is transferred from the first bottle to the second, in the same manner as the undiluted virus was taken, and the pipette with the residual virus suspension discarded as before. The second (1:100) dilution is mixed using a new pipette, and this serial operation is repeated to the last bottle. If a new pipette is not used for each dilution, the titration will be unreliable. Frothing, or the generation of

bubbles, should be avoided because results will be inaccurate.

Forty embryonating eggs incubated nine to 11 days should be candled for embryo viability and the edge of the air space margin marked with a pencil. All eggs should be marked with the titration number. Groups of five eggs should be marked from 10^{-5} to 10^{-10}. For controls, it is useful to add five or 10 additional eggs to the titration system and inoculate them with the sterile diluent.

The eggs should be inoculated as described previously with 0.1 ml per egg, using sterile 1-ml all-glass tuberculin syringes. Egg inoculation must start at the 10^{-10} dilution, and preferably a new syringe should be used for each dilution. The control eggs should be inoculated last. If during the procedure the operator should accidentally wet his hands with virus-containing fluid, he should wash and dry his hands before continuing the work. It is not necessary to use gloves, as this will add to the difficulty of controlling the syringe. However, the operator should wear a short-sleeved gown or a sterile coat with the sleeves rolled back to avoid contamintation by the cuffs of the coat.

Eggs with active embryos should be used for titration work. At nine days' embryonation, fertile eggs can usually withstand periods of one hour out of an incubator without any effect on viability.

Following inoculation, all eggs should be sealed as described in Chapter 5 and incubated at 37°C. The eggs should be candled daily, and any embryos dying within the first 24 hours should be discarded as non-specific mortality. Embryonic death during this period should not exceed 2 percent; common causes of death are embryos of poor viability, accidental haemorrhage caused by the inoculation, or bacterial contamination. If the death rate exceeds 2 percent, the cause should be investigated and corrected. After 24 hours' incubation the eggs should be candled twice daily, all dead embryos removed and the allantoic fluid checked for the presence of viral haemagglutinin.

Records should be kept of the virus titration being made so that the infected eggs may be marked as the embryos die. The incubation should be continued for seven days. All remaining eggs should be chilled overnight at +4°C and then examined for the presence of viral haemagglutinin. The estimation of the haemagglutinin content is too laborious to be used on individual eggs, and for spot testing titrations of infectivity, the following method should be adopted. A loopful of allantoic fluid from the egg on test is mixed with a loopful of a 10 percent suspension of washed chicken red blood cells on a white plate or a microscope slide. Haemagglutination is observed in about 15 seconds and its specificity can be checked by inhibition with specific Newcastle disease antiserum.

If the eggs are from a Newcastle disease-susceptible flock, it will be

found that over 95 percent of the infected embryos die before the expiry of the seven-day post-inoculation period. In many cases, the B1 or similar strains result in a death rate of 100 percent before the end of the seven-day post-inoculation period. With experience, the operator will become familiar with the death times, and as a result the frequency of candling may be reduced.

Calculating the end point. With groups of five embryos per dilution, a typical result will be as shown in Table 6.

TABLE 6. — TYPICAL TITRATION RESULT (FIVE EMBRYOS PER DILUTION)

Dilution						*Dead*	*Live*
—5	D	D	D	D	D	5/5	0/5
—6	D	D	D	D	D	5/5	0/5
—7	D	D	D	D		4/5	1/5
—8	D	D				2/5	3/5
—9						0/5	5/5
—10						0/5	5/5

The Spearman Karber calculation of the end point is given by the following formula:

$$m = x_k + \frac{1}{2} d - \frac{d \Sigma r_i}{n}$$

where: m = the end point
x_k = the log value of the last line of the titration (10)
d = the increment (log) — a tenfold increment is 10^1 (1)
Σr_i = the sum of all eggs in the titration that were not infected (14)
n = the number of eggs inoculated per dilution (5)

In the titration in Table 6 the values are:

$$m = 10 + \frac{1}{2}(1) - \frac{1\ (14)}{5}$$

$$= 10.5 - 2.8 = 7.7$$

$$EID_{50} = 10^{7.7}$$

For a more accurate titration involving an end point that can be predicted within limits, a preferable system is to make the initial dilutions in tenfold steps, and then to continue in fivefold steps over the expected end point. By reducing the dilutions to fivefold steps and by inoculating seven eggs per dilution instead of five eggs, a considerable increase in accuracy of the test will result (Allan and Hebert, 1968). For example, for an end point anticipated to be between 10^8 and $10^{9.5}$ the bench technique is as follows:

1. Fill seven Universal bottles with 9 ml diluent and mark them from — 1 to — 7.
2. Fill four more bottles with 8 ml diluent and mark them — 7.7, — 8.4, — 9.1 and — 9.8.
3. Dilute and mix the virus material as described previously up to and including bottle 7.
4. From bottle 7, remove 2 ml and transfer this volume to the bottle marked 7.7 (the addition of 2 ml to 8 ml gives a dilution of 1:5 for which the log value is $10^{-0.7}$).
5. Continue with the fivefold dilutions by serially transferring 2 ml up to and including the last bottle marked 9.8.
6. Mark and label 28 eggs and inoculate into the allantoic cavity 0.1 ml of dilutions — 7.7, — 8.4, — 9.1, — 9.8, using seven eggs per dilution. Seal the eggs, incubate and record the deaths. An example of a typical end point for this titration is shown in Table 7.

Using the Spearman Karber formula, the calculation for the example is as follows:

$$m = x_k + \frac{1}{2}d - \frac{d\Sigma r_i}{n} = 9.8 + 0.35 - \frac{0.7\ (14)}{7}$$

$$= 10.15 - 1.4 = 8.75$$

$$EID_{50} = 10^{8.75}$$

TABLE 7. — TYPICAL TITRATION RESULT (SEVEN EMBRYOS PER DILUTION)

Dilution								*Dead*	*Live*
—7.7	D	D	D	D	D	D	D	7/7	0/7
—8.4	D	D	D	D	D			5/7	2/7
—9.1	D	D						2/7	5/7
—9.8								0/7	7/7

By using this modified technique, a more accurate assay may be made of the viral content of batches of vaccine and the small loss in viability associated with freeze-drying.

The experimental error of a titration is a combination of the chance pattern of death in the inoculated eggs, the precision with which the operator carries out the test, and the accuracy of the pipettes. Thus, as indicated by Allan and Hebert (1968), it is not possible to quote expected errors of mean. These authors gave results based on the titration by three different operators according to the methods outlined above. The standard error of mean for a full tenfold dilution series was about ± 0.3. Duplicate titrations increased the accuracy of the estimation.

Fresh, correctly harvested amnio-allantoic fluids from eggs infected with the lentogenic strains of virus can be expected to contain between $10^{10.0}$ and $10^{10.3}$ EID_{50} per ml. Mesogenic virus will be found in slightly lower concentrations ranging from $10^{9.8}$ to 10^{10} EID_{50} per ml.

With lentogenic vaccines, the optimum range on the dose response scale is about $10^{7.0}$ EID_{50} per bird. This optimum range will vary with the vaccine under test, but is the standard recommended.

With minimal losses on storage and freeze-drying, 1 ml of fluid will yield a vaccine that contains 1 000 doses of $10^{7.0}$ EID_{50}. In general, virus loss will be greater, and in order to attain the optimal immune response the vaccine vials should be filled with 2 ml of allantoic fluid for each 1 000 doses.

It is essential that vaccines retain a satisfactory potency during a storage period of one year. To determine this potency, an accelerated stability test should be carried out. In general, storage of freeze-dried vaccine at 37°C for 12 days may be taken as equivalent to storage at — 20°C for one year.

Alternatively, storage of freeze-dried vaccine at 37°C for three to five days may be equivalent to storage at +4°C for one year. Other results indicate that one-week storage at 37°C was equivalent to more than two years' storage at +4°C (Simi *et al.*, 1970).

The vaccine should be produced so that a bird dose of not less than $10^{6.5}$ EID_{50} will be present after storage for one year or the equivalent titre on the accelerated test. For further details, see Appendix 9.

The haemagglutination inhibition test

Studies are in progress to standardize the haemagglutination inhibition test in order to eliminate the serious differences which occurred in previous years. The 1963 and 1971 editions of *Methods for the examination of poultry biologics* (National Academy of Sciences, 1971) defined both the constant serum, varying antigen method (alpha procedure) and the constant antigen, varying serum method (beta procedure). These publications suggested that the activity of the serum sample could be given as the serum dilution reciprocal times the antigen content, which caused complete inhibition of haemagglutination. Allan and Gough (1974a) suggested the adoption of the beta method using 4 HA_{50} of virus antigen with the expression of the result as the reciprocal of the 50 percent end point of the serum dilution.

Isolation and identification of avian pathogens (Hitchner, 1975) reviews the methods in use and suggests that the beta method of serology be used and the result expressed as the reciprocal of the serum dilution, and not (as previously) as the multiplication of the antigen strength and the serum reciprocal. The methodology is now better standardized, although lack of complete international agreement results in varying methods of expression of HI titres.

At present, the general recommendation is that the Newcastle disease HI test be carried out by the beta method, that is, constant antigen and doubling dilutions of serum. The methodology described in the present publication has been compared with that reported by Hitchner (1975). The studies conducted have included the use of the international Newcastle disease reference serum which is available for laboratory standardization purposes.

Source of haemagglutinin. Any strain of Newcastle disease virus may be used for the production of haemagglutinin. The added safety of using inactivated virus is to be preferred, although live virus can be used. Lentogenic virus is preferable to velogenic virus, partly because the former usually grows to a higher titre in embryos, and partly because lentogenic vaccine strains present a less dangerous hazard in the laboratory. Strains

commonly used for the production of haemagglutinin include the B1, La Sota and Asplin's F strain. At the Central Veterinary Laboratory at Weybridge, the F strain has been used because of its good keeping qualities which provide added stability and reproducible results in the test.

It is not necessary to use SPF eggs for the production of haemagglutinin, although eggs that are free from Newcastle disease antibodies should always be used.

Inoculation of not less than 10^2EID$_{50}$ and not more than 10^4EID$_{50}$ per 0.1 ml into the allantoic cavity of nine-day embryonating eggs will result in high virus titres. The eggs should be candled twice daily and all embryos dying during the first 24 hours discarded. The eggs should be chilled just before the mean embryo death time (about 96 hours for lentogenic virus) and the amnio-allantoic fluids (AAF) harvested aseptically, taking care to avoid contamination with albumen or yolk.

Inactivation can be carried out at 37°C with 1:1 000 formalin, which will generally result in a more stable HI antigen than BPL. The antigen should then be tested for lack of infectivity and stored.

Bulk haemagglutinin will retain its initial titre of 2 048 to 4 096 at +4°C for several months provided that it is kept sterile. If stored at temperatures from — 15 to — 40°C, there will be a gradual loss in titre over a period of months, and where antigen has been divided into small working lots this drop in activity may be erratic. It has been found that by dividing the haemagglutinin into working lots of 5 ml and storing these at — 68°C, the titre has been maintained for periods of over three years, thus giving a more reproducible test system. The addition of thiomersalate at 1:10 000 may help reduce contamination.

Preparation of the working haemagglutinin. Undiluted allantoic fluids generally have a titre of between 512 and 2 048, and therefore the fluid must be diluted to give the required 4 HA units. For an accurate end point, the HA$_{50}$ is chosen. This is defined as that level of antigen which causes 50 percent haemagglutination. A 50 percent HA is less than a maximal HA (100 percent agglutination) and will therefore be more easily inhibited and produce a numerically greater HI titre.

To obtain an accurate 4 HA$_{50}$, the suspension should be tested in two ways. The first step is to prepare a series of twofold dilutions of the undiluted fluids to determine the activity, for example, 1 024 or 2 048. The fluid should then be diluted to give a theoretical 4 HA, that is, $\frac{1\,024}{4}$ = 1:256. This dilution can be prepared by adding 1 ml of AAF to 255 ml of isotonic saline. A more accurate procedure is to add 1 ml of AAF to 9 ml of saline to make an initial 1:10 dilution, and a further dilution is made by adding 1 ml of the 1:10 to 24.6 ml of saline to produce a

final dilution of 1:256.

The second step is to check the accuracy of the 4 HA_{50}; this is done by preparing a series of dilutions in tubes by adding 1 ml of the 1:256 suspension to 1 ml, 2 ml, 3 ml, 4 ml, 5 ml and 6 ml, giving final dilutions of haemagglutinin of 1:2, 1:3, 1:4, 1:5, 1:6 and 1:7.

Finally, 0.2 ml of each viral suspension is added to 0.2 ml of a 1 percent suspension of triple-washed chicken red blood cells (RBC) and a final 0.2 ml of physiological saline added to each well of the WHO plate. The plate should then be shaken backward and forward and from side to side to mix the virus and red cells. Alternatively, the microtest can be employed and 0.05 ml of the antigen suspension added to 0.05 ml of a 1 percent chicken RBC suspension and 0.05 ml physiological saline added as blank (the final 0.05 ml saline blank takes the place of the 0.05 ml of diluted serum which would be used in the HI test and keeps the test at standard volume) (Picault *et al.*, 1975).

The plates should be held at bench temperature (20 ± 2°C) for 35 to 40 minutes for the large WHO plates (or a somewhat lesser time for the microplates, which should be of the round-bottomed variety) until a clear pattern of haemagglutination is seen. The HA_{50} is that dilution in which a layer of cells settles out from half the red blood cells and the other half gathers in a round button at the centre of the plate. If the original suspension is confirmed as giving exactly 4 HA_{50} from a 1:256 dilution, then the 1:4 sample will give the 50 percent end point. If the HA is higher than 4, then the 1:5 or 1:6 will give the end point, and if lower, then the 1:3 or 1:4 dilution is used. Should the 1:5 dilution be found to be the best end point, then the HI tests would be carried out by diluting the antigen $\frac{5}{4}$ times 256 = 1:320.

Many workers conduct the HA and HI tests for Newcastle disease with unpurified inactivated AAF; however, Beard *et al.* (1975) have reported the advantages of standardization and relative stability of a polyethylene glycol (PEG) concentrate.

Preparation of red blood cells. The method of HI testing at present recommended specifies a 1 percent RBC suspension. The best results are obtained by taking blood from at least four different chickens which are 2 to 6 weeks old and fully susceptible to Newcastle disease. Blood should be taken by hypodermic syringe into Alsevers solution. The formula for Alsevers solution is:

Dextrose	20.5 g
Sodium citrate	8.0 g
Sodium chloride	4.2 g
Distilled water	1 l

The pH is adjusted to 6.1 with a newly prepared 10 percent solution of citric acid. The red blood cells are washed three times by gentle centrifugation in physiological saline (1 500 rev/min for five minutes). The final suspension can be stored at +4°C for several days according to the degree of contamination. When the cells become dark in colour they should be discarded. In practice, collecting blood twice a week gives a constant supply of cells.

To prepare a 1 percent suspension, 1 ml of gravity-deposited RBC should be mixed with 99 ml of physiological saline (phosphate-buffered saline is not necessary, and may give results less clear than tests with unbuffered saline because the phosphate may interfere with the test). This suspension of RBC is checked for concentration by a haematocrit test. To conduct this test, 60 ml of the nominal 1 percent suspension are measured and allowed to sediment. Fifty ml of saline are removed to give an apparent 6 percent suspension; this is mixed thoroughly and a standard haematocrit tube is filled and centrifuged at 10 000 rev/min for five minutes. The packed cell volume should be exactly 6 percent.

After the RBC concentration is determined for the first time, the 1 percent suspension can be read on a colorimeter using a red filter. Subsequent suspensions are adjusted to give exactly the same deflection. Using an EEL colorimeter and 1-cm diameter tubes, a 1 percent chicken RBC suspension has been found to give a deflection of exactly 50 percent.

Chicken RBC should be used for tests with chicken serum, turkey cells with turkey sera and so forth. When it is not possible to match the RBC species and the serum donor species, it is best to adsorb the test sera with equal volumes of 10 percent chicken RBC before the HI test is conducted.

Collection of serum. Serum is best collected with a hypodermic syringe from the wing vein and by placing about 1 ml of whole blood in a tube to give a height approximately equal to the tube diameter. This will give better serum separation than samples taken into long slim tubes. Clotting and subsequent serum separation take place more rapidly if the samples are held at about 37°C for several hours and when each sample is " ringed " by running a platinum wire round the edge of the clot to free it from the wall of the tube. Samples kept warm and ringed well may have the serum removed after a few hours. For other samples, the serum and clot are best left overnight before the serum is removed. Care should be taken to avoid collecting free RBC with the serum sample. It is important that samples arrive in the laboratory in good condition. Serum samples should only be dispatched after the blood clot has been removed, and if transit is likely to be long and subject to temperatures above 20°C the samples should be sent refrigerated. On receipt, all samples should be stored at +4°C or lower until tested. Any serum samples that come

from haemolysed blood samples or are visibly contaminated should be discarded; otherwise false test results may occur.

Non-specific inhibitors. The non-specific inhibitors in chicken sera seldom have a titre of more than 1:4 to 1:8, and about 60 percent of this activity can be removed by heat inactivation in a water bath at 56°C for 30 minutes. All samples should be treated in this manner. Other methods for the treatment of non-specific inhibitors include periodate treatment and the receptor-destroying enzyme (RDE) treatment. Reference to the methods of treatment may be found in U.S. Department of Health, Education and Welfare (1975).

Haemagglutination inhibition test methods. This test can be carried out on WHO plates with 0.2-ml aliquots or in microplates with 0.05- or 0.025-ml aliquots. A common method involves the use of the round-bottomed microplates, usually with 0.05-ml aliquots. It should be noted that as the principle of the test is that of equivalence between antigen and antibody, the volumes used do not interfere with the test, provided they are the same.

For using the WHO test plate, 1-ml white-backed glass pipettes controlled by rubber teats are recommended. For the microtests, Takatsy microloops (Figure 14) are used. These may be operated manually (Beard and Wilkes, 1973) one at a time, or in sets of eight, or may be used with automated machines such as the Canalco autotitre Mk IV, or one of the Titretek machines, in which the Takatsy loops lower, mix and carry over mechanically.

Allan and Gough, (1974a, 1974b) have found that the automated methods give more reproducible results, and for large numbers of tests the use of machines which prevent operator fatigue is recommended.

Plates with eight rows of 12 columns of wells allow eight samples to be titrated from 1:2 to 1:4 096 (2^1 to 2^{12}). To test within this range, each well of the whole plate should be filled with

FIGURE 14. Takatsy microloops for the haemagglutination inhibition test
(Photo courtesy Cook Instruments, Ltd., United Kingdom)

the standard volume of physiological saline (0.05 ml for the usual micromethod or 0.2 ml for the WHO macromethod), and eight serum samples placed in the first row of wells. After mixing, the standard volume is carried over to the second row and the procedure repeated until the twelfth row, when the standard volume is discarded (of if further dilutions are to be tested, the standard volume is carried on to a second plate to cover the range from 13 to 16 which represents the limit of known HI titres).

After the serum has been diluted in the wells of the plate, 0.2 ml of 4 HA_{50} antigen is placed in each well. For the WHO test, 15 minutes should be allowed for antigen-antibody reaction. For the microtest, it has been found that the reaction time is so short that a specific time need not be allowed and the RBC suspension can be added directly and the plates agitated backward and forward and from side to side to ensure an even suspension of RBC in the mixture. The WHO plates are then left for 40 minutes, or 30 minutes in the case of microplates, or until a clear pattern of haemagglutination has appeared. It is a mistake to read the test too soon; it is preferable to leave the plates for a longer time period. During the sedimentation the plates are kept at room temperature (20-22°C).

The end point is taken as the well in which haemagglutination is partly (50 percent) inhibited, i.e., the first well in which there is not a clear button of red cells and in which it is judged that 50 percent haemagglutination has occurred. This latter is seen as a wide deposition of red cells over the bottom of the well.

It is sometimes useful to tilt the plate slightly to one side to allow non-agglutinated cells to roll to the side. The agglutinated cells will remain in place.

Different operators may tend to judge the end point at different dilutions, and it is important that all workers in one laboratory compare their readings of the test in order to obtain a standard procedure.

The HI titre is expressed as the reciprocal of the dilution of serum in the well taken as the end point. In a twofold series this can be expressed as 16, 32, 64, 128, etc., or in $\log_2$ notation as 4, 5, 6, 7 respectively.

When the first well in the tray has been diluted 1:2, the titre at that well is recorded as 1, that is, 2^1, hence the titre of the sample is taken by counting the number of wells from the beginning of the test to and including the end point well. In a dilution series where serum is first diluted 1:5 and then in twofold steps, the values in succeeding wells are 10, 20, 40, 80, 160. As these dilutions cannot be easily referred to $\log_2$ notation, they must be transformed into $\log_{10}$ values for geometric mean titre calculation. With the twofold system it is usual to refer to the titres in the $\log_2$ form and not to transform them back by the use of anti-$\log_2$. For accurate work, reports should include a check test with the international reference serum.

The relationship between haemagglutination inhibition levels and challenge. Experimental challenge with an injection of 10^6ELD$_{50}$ of Herts '33/'56 has been found to result in a death rate similar to that occurring in field outbreaks during the 1970-71 epizootic of highly virulent virus in the United Kingdom. In the laboratory this challenge can be summarized as follows:

All individual HI values 2^2 or less	=	100% mortality on challenge.
Range 2^2 to 2^5 log mean $2^{3.75}$ s.d. 0.4	=	10% mortality on challenge.
Range 2^4 to 2^6 log mean $2^{5.2}$ s.d. 0.35	=	0% mortality on challenge.
Range 2^6 to 2^8 log mean $2^{6.5}$ s.d. 1.2	=	Serious drop in egg production, no deaths, and convalescent HI titre 2^{14} or greater.
Range 2^9 to 2^{11} log mean $2^{10.5}$ s.d. 1.4	=	No drop in egg production, no deaths, convalescent titres 2^{11} to 2^{12}.
Range 2^{11} to 2^{13} log mean $2^{11.2}$ s.d. 1.3	=	A flock that will remain free from the risk of a drop in egg production for more than six months.

NOTE: s.d. = standard deviation.

International Newcastle disease reference serum. This preparation is available in limited quantities from the WHO/FAO International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, United Kingdom, for the purpose of standardizing laboratory reference sera. Reconstituted to 1 ml, the serum has been found to give a titre of 2^6 (1:64) using the HI system described above.

The method used by the American Association of Avian Pathologists. In the American Association of Avian Pathologists' publication *Isolation and identification of avian pathogens*, Hitchner (1975) discusses the difference between alpha and beta HI test methods and describes the methods for an HI test based on the tube method. A chapter on microtest methodology is also given. The tube method is based on an HA level of eight minimal units as follows:

HAEMAGGLUTINATION INHIBITION TEST

Reagents (ml)	Tube [1]						
	[2] 1	2	3	... 8	9	10	[3] 11
Saline	0.8	0	0	0	0	0	0.5
16 HA antigen	0	0.5	0	0	0	0	0
8 HA antigen	0	0	0.5	...0.5	0.5	0.5	0
Test serum	0.2	0	0	0	0	0	0
Transfer	0.5 →	0.5 →	0.5	... 0.5 →	0.5 →	0.5	
0.25 % RBC	0.5	0.5	0.5	0.5	0.5	0.5	0.5
						(Discard 0.5)	
Serum dilution	1:5	1:10	1:20	1:640	1:1 2800	1:2 560	

[1] Each tube has a final volume of 1.0 ml of which 50 percent comprises the RBC suspension and half a dilution series of serum together with 8 HA units of antigen. — [2] Serum control. — [3] Saline RBC control.

In a comparative test using the FAO method and the method of the American Association of Avian Pathologists (AAAP) simultaneously, test sera gave the following results:

Method	Sera							
	1	2	3	4	[1] 5	[1] 6	[2] IRS	[2] IRS
FAO	2^{10}	2^{10}	2^{9}	2^{9}	2^{2}	2^{3}	2^{6}	2^{6}
AAAP	2^{9}	2^{9}	2^{8}	2^{8}	2^{2}	2^{2}	2^{6}	2^{6}

[1] Newcastle disease-negative serum. — [2] International Newcastle disease reference serum.

It can be seen that the FAO method reads one tube higher at high levels. This would be expected from the use of smaller amounts of antigen. The United States method gives clearer negative results than the FAO method; however, both give a value of 2^{6} for the international reference serum.

The potency test in chickens

Chickens used for the potency testing of a Newcastle disease vaccine must be produced from a parent flock that is free from the disease and has not been vaccinated against it. This is necessary in order to provide fully susceptible birds. In countries where the disease is enzootic, a source of susceptible chickens may be difficult to find. Susceptible chickens are essential if the following test procedures are to be of value.

If susceptible stock cannot be produced locally, then consideration should be given to importing fertile SPF eggs and rearing the hatched chicks under conditions of adequate disease security. Test results may vary with the type of bird used, and every effort should be made to maintain a supply of uniform test chickens.

It is advisable to immunize birds at 3 weeks of age. Post-vaccination blood samples and challenge should be carried out 21 days after vaccination. Test groups should include not less than 15 birds so that the variation in individual response may be estimated. It is seldom necessary to have more than 25 chickens per group. Control groups of not less than 10 chickens should be maintained.

The control chickens should be from the same source and hatch as the test birds, but they should be kept in separate units until the challenge tests are carried out. This isolation procedure will assist in keeping the birds free from vaccine virus.

Blood samples from 10 percent of the birds should be taken before any test procedure is begun. An HI test on the sera is necessary to ensure that the birds have no Newcastle disease antibodies.

For testing live vaccines, one field dose and one tenth of a field dose are usually administered. Application of the vaccines may be by the ocular route, the drinking water route or by intramuscular injection. The vaccination route used should correspond to the usual route of field application in order to avoid a false impression of vaccine potency.

The challenge virus. The results depend on the strain of virus used, the passage history, laboratory storage conditions, dose and route of administration.

The standard British challenge for inactivated vaccine is an injection of 10^{6} EID_{50} of the Herts '33 strain per bird. In other countries the GB Texas strain has been used at $10^{4.0}$ to $10^{6.0}$ ELD_{50} per bird. Intramuscular challenge provides the most reproducible lethal effect in fully susceptible birds. Challenge by contact or by intranasal application of virus is considerably more erratic in effect. Challenge by aerosol spray of lethal pneumotropic strains of virus provides a severe challenge. The aerosol spray method is more difficult to reproduce because it involves the control of a number of

factors including temperature, relative humidity, droplet size, ventilation rates and density of birds. Therefore, it is recommended that challenge estimations be confined to the intramuscular injection of well-characterized laboratory strains of virulent virus. The challenge strains commonly used are Herts '33, GB Texas and Milano. Of these, the Milano strain is the most lethal and the GB Texas the least.

It is important to note that with repeated laboratory passage and storage, all these strains may lose virulence. Hence it is recommended that before storage as challenge seed viruses, they be subjected to three passages in chickens using splenic material (Chapter 3). Where it is necessary or considered important that a local field strain be used in place of the above challenge strains, it should be realized that the local strain may be of greater or lesser potency. Hence by using a local strain only, comparative potency results between countries cannot be firmly established.

Before use, a challenge strain should be titrated in chickens to establish the chick-lethal dose (CLD_{50}). No strain should be used for challenge unless the CLD_{50} is less than $10^3 ELD_{50}$.

Potency determinations

Lentogenic vaccines. To assess potency, a lentogenic vaccine should be administered to four groups of 20 three-week-old chickens as follows:

Group 1. One field dose per bird in 10 ml drinking water.
Group 2. One tenth of a field dose in 10 ml of drinking water.
Group 3. One field dose in 0.05 ml physiological saline by the ocular route.
Group 4. One tenth of a field dose in 0.05 ml physiological saline by the ocular route.

Alternatively, the specifications given in United Kingdom (1976) may be used.

Control chickens are required, and these should be housed in isolation and separated from the immunized groups. Individual blood samples should be taken from all birds three weeks after immunization. The vaccinated and control chickens are then challenged by intramuscular injection.

Potent vaccines of the lentogenic type should provide full protection following one field dose administered by the ocular route. Vaccines with a high viral content should give 90 percent protection when one tenth of a field dose is administered by the ocular route. Drinking water administration will result in less protection. Poor handling of the vaccine strain of virus may reduce the protection afforded by the drinking water route to

less than 50 percent, even at a full field dose. Vaccines of low potency should not be considered satisfactory for drinking water application.

Determination of the HI levels of identified birds can provide a correlation between the HI value and the challenge results. When using the HI test, it is important that a standard laboratory reference serum be included in each test (Chapter 7). Unless this is done, the HI results may vary between tests. It is convenient to prepare the reference serum with an HI value between 1:64 and 1:128 (2^6 and 2^7).

The HI method, when compared with challenge of vaccinated birds using $10^{6.0}ELD_{50}$ of Herts '33 strain per bird, gives a mean HI value of birds dying of 1:9 ($2^{3.2}$).

The correlation between challenge results and the HI values will allow a more complete estimate of the potency of the vaccine. A satisfactory vaccine will give a close correlation and a high degree of resistance to challenge. At the time of issue, vials of vaccine should contain more than the minimal viral content. Therefore, the results of the tests with one tenth of the field dose should be not less than 80 percent protection after drinking water administration.

In laboratories where the use of challenge virus presents a problem of isolation, it is suggested that the challenge evaluation be conducted at widely spaced intervals, and for routine batch testing a serological assessment of the vaccine be used.

Mesogenic vaccines. Potency tests for mesogenic vaccines are not easily carried out by immunization and challenge because the high immunogenicity of these vaccines makes the determination of any useful end point a matter of some difficulty. It may be preferable to estimate the potency of these vaccines by an evaluation of the HI response.

Work with several different isolates of the Komarov strain at the Central Veterinary Laboratory at Weybridge showed that they could have an intracerebral pathogenicity index (ICPI) of between 1.1 and 1.4. None of the strains examined were found to be free from spreading, and in passage in chickens all became contagious, thus rendering them dangerous for use on premises where young chickens were kept with older birds.

The most effective method of administration was found to be by the intramuscular route, when a group of 20 four-week-old chicks had a mean HI response of $2^{8.35}$ 21 days after being inoculated with $10^{7.0}ELD_{50}$ of virus, while in the same test the international standard serum gave a value of 2^6. This can be interpreted to mean that mesogenic vaccines of the Komarov type can be expected to give log mean HI values of from two to four times that of the international standard preparation.

Potency tests of these vaccines by dilution steps will be of little use, as in the fully susceptible chick small amounts of virus may still exert a consid-

erable immunizing effect, while as a secondary vaccine (the normal manner of use of these vaccines) the virus content may have to be much higher. It is not practical to test these vaccines in terms of the secondary response, because the response then measured will be partly a function of the sensitizing dose, the time between the two doses, and the quality of the secondary vaccine itself.

Inactivated vaccines. A potency test consisting of an injection of a fixed amount of virulent virus (Herts '33/'56) into birds three weeks after they have received a full dose of inactivated vaccine can be used. The vaccine is approved for use if all vaccinated birds survive the challenge. Alternatively, a graded vaccine dose test can be used in which that dilution of the vaccine giving 50 percent protection is determined (PD_{50}).

For aqueous vaccines incorporating aluminium hydroxide as an adjuvant, the standard 0.5-ml vaccine dose is injected in physiological saline dilutions of 1:50, 1:100 and 1:200 into groups of three-week-old chicks using 15 per group. Three weeks later, all birds including five non-vaccinated controls are injected with $10^6 ELD_{50}$ of low-passage Herts '33/'56 challenge virus. The number of birds dying is recorded for a 10-day period.

The PD_{50} value with 95 percent fiducial limits is calculated by probit analysis, and only vaccines which exceed a defined minimum potency value are passed for use.

The test for oil emulsion inactivated vaccines is similar, except for the use of reduced dose volumes instead of vaccine dilutions. This is conducted by administering the vaccine using a micrometer syringe. For vaccines of this kind, the PD_{50} values have been generally well in excess of 100 PD_{50}. The doses tested have included 1/50th, 1/100th, 1/200th and 1/400th parts of a 0.5-ml dose. This allows the calculation of higher PD_{50} values.

Using AAF as a basis for aluminium hydroxide adsorbed inactivated vaccines, potency values from 50 to over 100 have been recorded. Based on inactivated AAF, oil emulsion vaccines have shown potency values of over 200.

Other test requirements

The following is a guide in the design of purity tests. Further details are given in Appendix 6 and in the publication *Methods for examining poultry biologics* (National Academy of Sciences, 1971).

Salmonellae. This can be based on a 5-ml sample of the liquid vaccine suspension added to 100 ml of liquid broth medium. The liquid vaccine

suspension should not contain antibiotics or other bacteriostatic or bactericidal agents.

Extraneous bacteria, fungi and yeasts. Five final container samples (either liquid vaccine or freeze-dried vaccine rehydrated with sterile distilled water) should be cultured separately in nutrient agar poured plates.

Mycoplasma species. One sample of the final product is cultured in mycoplasma media.

Lymphoid leucosis virus. This can be based on at least one 3-ml sample of the bulk harvest taken before the addition of antibiotics or other inhibitors. The COFAL (complement fixation for avian leucosis) test is recommended (Koski *et al.*, 1970).

Extraneous pathogens. Final container samples of the completed product should be examined for extraneous pathogens by either the chick embryo inoculation test or by the inoculation of Newcastle disease-immune chickens.

Safety tests in young chickens. These tests are conducted to determine the safety of the vaccine in susceptible young chicks.

Virus content using chick embryos. See the section on the embryo-infective dose 50 percent end point.

Immunizing capability. See the section on the potency test in chickens.

8. VIRUS CONTENT OF VACCINES

The concentration of virus will depend on the type of vaccine being produced and on the immunogenicity of the substrain used for production. The potency of inactivated vaccines is dependent primarily on the concentration of antigen in the vaccine, but the potency is affected by the type and efficiency of the inactivation process and by the kind and concentration of adjuvant with which the antigen is combined.

The efficiency of the manufacturing process can be determined by accurate monitoring of the viral content at each stage of production. The value of the monitoring procedures depends on the accuracy of the viral assay systems conducted by the laboratory control section. Thus considerable attention should be paid to the development of assay techniques that are both accurate and reproducible.

Lentogenic vaccines

As discussed in Chapter 7, the yield of virus from amnio-allantoic fluids infected with a B1 type of virus can be expected to average $10^{9.25}EID_{50}$ per 0.1 ml. One ml of this fluid will yield 1 000 doses, each containing $10^{7.25}EID_{50}$. In studies of the dose response curve in three-week-old susceptible birds, the optimal dose of virus was between $10^{6.5}$ and $10^{7.0}EID_{50}$ per bird.

The objective of the production unit should be to attain these levels of virus in the issued vaccine. The factors affecting the virus content include the losses involved in the freeze-drying process and on storage of the amnio-allantoic fluids prior to drying. Low concentrations of harvested virus also cause problems.

To ensure the optimal concentration of virus in the finished product, an effort should be made to assay the virus content at each stage of the production cycle.

Possible causes of low yields of harvested virus

1. The use of embryonated eggs from flocks having antibody to Newcastle disease. This antibody may reduce the amount of virus released into the embryonic fluids.

2. In the harvesting process the accidental inclusion of small amounts of yolk may cause neutralization of some infective virus.
3. Contamination of the virus fluids with albumen may also affect the concentration of virus.
4. Inoculation of seed virus that has been passaged at high multiplicity may result in significantly lower virus yields (Appendix 3).
5. For each vaccine virus strain the time of incubation during which the virus infectivity titre reaches its highest level in the inoculated eggs is determined, and at this time these eggs are removed and chilled. Because most of the embryos inoculated with lentogenic strains should remain alive at the optimal harvest time, many institutes harvest only the living embryos after chilling. If dead embryos are harvested, these should be checked separately for the EID_{50}.
   Chilling of the embryos immediately after incubation is very important, because haemorrhage from live unchilled embryos will cause contamination of the AAF with red blood cells.

Possible causes of low virus content prior to freeze-drying

1. Freezing, storage and subsequent thawing of the bulk virus can cause appreciable losses of infectivity. Thus, fluids should be chilled but not frozen, and held at +4°C. Every effort should be made to freeze-dry the vaccine within 24 hours of the virus harvest; that is, before the final results of the safety and bacterial sterility tests are known.
2. Contamination of the virus fluids with disinfectant solutions used in the laboratory can have serious effects.

Losses during freeze-drying. Different types of freeze-drying equipment are operated in different ways (see Appendix 4). As the storage time is largely dependent on the efficiency of the freeze-drying process, it is strongly recommended that the freeze-drying machine be fitted with a means of recording the temperature and pressure during the production of each batch of vaccine. Lack of maintenance of the equipment, in particular the lessened efficiency of the condenser system or of the vacuum pump, may lead to a gradual decline in efficiency. Unless monitored, equipment that produced a stable product initially may later give rise to a less stable vaccine.

Losses during storage. It is recommended that vials of vaccine be stored at −20°C prior to issue. The temperature of storage refrigerators should not be allowed to fluctuate widely. The loss of potency after freeze-drying is closely associated with the residual moisture content of the vials. In certain

circumstances when the residual moisture is below 1.7 percent, vials may be stored at +4°C.

Losses during transport and local storage. Small quantities of vaccine dispatched by normal systems of transport may become heated during transit. The viability of the vials at 37°C should be estimated and transport should be arranged to ensure that vials are held at ambient temperatures for considerably less than the period known to cause a detectable drop in viability.

Filling level of vials. Tests on the virus content of harvested fluids before and after freeze-drying will permit an accurate estimate to be made of the viral content of the final vaccine. Losses during freeze-drying of $10^{0.5}$ to $10^{1.0}EID_{50}$ are common.

Where the losses during freeze-drying are as low as $10^{0.25}EID_{50}$ and where wet fluids are found to have a content of $10^{10.25}EID_{50}$ per ml, one vial containing 1 ml of amnio-allantoic fluid will contain at issue $10^{7.0}EID_{50}$ per dose (assuming 1 000 doses per vial). These figures represent the levels that can be obtained under optimal conditions, but may be difficult to achieve under certain circumstances. By increasing the vial content to 2 ml, the titre will be raised by $10^{0.3}EID_{50}$, and in many laboratories this volume will be the most practical.

Allowance should be made for the decrease in virus content during storage. A loss of $10^{0.5}EID_{50}$ or more, following storage of one year, should be considered unsatisfactory. Vials should be labelled with an expiry date not more than one year from the date of manufacture, which should be taken as the date of freeze-drying. If stability of the vaccine is less than the above figure, then the expiry date should be shortened accordingly.

Mesogenic vaccines

An infectivity dose of $10^{5.0}ELD_{50}$ per bird is usually considered adequate for mesogenic vaccines. This means that 1 000 dose vials can be produced from 1 ml of allantoic fluid. However, it is more difficult to harvest mesogenic vaccine virus at a titre of $10^{8.8}$ to $10^{9.0}ELD_{50}$ per 0.1 ml. It is also difficult to time the harvest of the mesogenic vaccine strains in order that the eggs may be removed from the incubator close to the average embryo death time. Eggs which remain in the incubator after embryo death begin to autolyse. This can be avoided if a close study is made of the average death time of the vaccine strain being used. The death time will be affected by the accuracy of the incubator temperature. Cold areas in the incubator will lead to longer death times. Unusually small eggs will tend to die more

quickly than standard-size eggs. The death time will also be affected by the amount of virus inoculated into each egg. Hence the techniques of viral dilution and inoculation should be maintained in a standard manner. The addition of excess virus to the inoculum will lead to accelerated death times with correspondingly reduced viral content of the harvested fluids.

9. TESTS FOR VIRUS CHARACTERISTICS

Newcastle disease virus isolates do not vary in terms of the antigenic component. The ability to agglutinate mammalian red blood cells and the heat stability of the haemagglutinin at 56°C have been used in the characterization of individual strains. However, these tests have not been found useful in the general classification of isolates. The pathogenicity of the virus is dependent on its activity and on the susceptibility of the embryonated eggs or chickens used in the tests. Thus for this work, all eggs and chickens must be from a flock fully susceptible to Newcastle disease. For replicate tests, it is best to use birds from the same source in order to obtain consistent results. In tests with chickens, the value or index obtained depends on recording numerically the severity of reaction to the virus. The birds must be classified as normal, sick or paralysed. This classification is subjective, and therefore it must be appreciated that the score or numerical recording can vary with the interpretation made by the observer.

Pathogenicity tests

The three tests commonly used are the mean death time (MDT) in embryonating eggs, the intracerebral pathogenicity index (ICPI) in day-old chicks, and the intravenous pathogenicity index (IVPI) in six-week-old chickens. These tests are the generally accepted means of assessing the virulence of Newcastle disease isolates (Martone *et al.*, 1973, 1974). Technical details are given in the publication *Methods for the examination of poultry biologics* (National Academy of Sciences, 1971). The following section is based on that publication.

Mean death time. This is the time in hours required for the minimum lethal dose (MLD) to kill 9- to 11-day embryonating chicken eggs.

Tenfold dilutions of fresh infected allantoic fluid that has been recently harvested, or stored at +4°C for not more than one week, are made in sterile physiological saline containing antibiotics. Dilutions are from 10^{-1} to 10^{-9}. Thirty 9-day embryonating eggs of standard size are candled and

marked. Small eggs die slightly earlier than large eggs, and a variation in egg size will make the test less reproducible. Ten eggs are marked for each dilution in the series 10^{-7}, 10^{-8} and 10^{-9}. Five eggs are marked " A " for the morning inoculation and the remainder " B " for the afternoon inoculation.

At 8 a.m. or earlier, groups of five eggs are inoculated in the allantoic cavity with 0.1 ml of each dilution. The hole in the shell is sealed and the eggs are incubated at 37°C. Following these inoculations, the virus dilutions are maintained at +4°C, and late in the afternoon (5 p.m.) the " B " series of eggs are inoculated. All eggs are candled twice daily at 8 a.m. and 5 p.m. The time of death of each embryo is recorded. Eggs with dead embryos should be examined for the presence of Newcastle disease haemagglutinin (see Chapter 7).

The test is continued for seven days, and at the end of this time all remaining eggs are chilled and tested for haemagglutinin.

The minimum lethal dose is the highest dilution at which all the embryos in both sets of eggs die, and the mean death time of these embryos is recorded. The test may be made more accurate by increasing the number of eggs from five per dilution to 10. However, in practice the difference in the results is minimal.

When a series of these tests are to be carried out, a modified technique may be used. In this technique, the allantoic fluid is diluted to 10^{-8}, using four hundredfold dilution steps. These dilutions are obtained by adding 1 ml virus fluid to 99 ml of physiological saline solution. The suspension is mixed and three further dilutions are prepared in a similar manner.

The resulting 10^{-8} dilution is inoculated into two sets of seven eggs: a morning set and an afternoon set. The mean death time is calculated from all the embryos that die. In practice, this modified technique has given results very similar to those obtained with the more usual method.

The test is accurate only when the eggs do not contain antibody to Newcastle disease virus. In the case of velogenic virus, with a short mean death time (less than 60 hours), eggs containing Newcastle disease antibody cause little error. However, in testing lentogenic strains, the effect of passive antibody in the egg is more pronounced.

Intracerebral pathogenicity index. 0.05 ml of fresh infected allantoic fluid diluted 10^{-1} is injected intracerebrally in each of 10 one-day-old chicks. The saline diluent must be sterile, but should not contain antibiotics. The virus dilution under test should be plated out for bacterial sterility, and the test is valid only if the inoculum is free from bacteria. It is useful to include two additional chicks inoculated with the sterile diluent only. The inoculations should be carried out with a fine hypodermic needle (a needle 0.45 mm in diameter and 5 mm in length is suitable). The location of the

inoculum in the brain is not important. However, inoculation from the caudal aspect of the cranium is easier than injection from the anterior aspect.

The test is read daily by inspection of the chicks at the same hour of the day as the original injection. Chicks are scored or rated as normal (alert, moving without incoordination), sick (including birds that are exhibiting signs of paralysis or are prostrate, but excluding chicks that are only dull), and dead. Care must be taken in distinguishing chicks which are being incorrectly brooded and are thus disinclined to move, from chicks that are affected by the virus. In making these comparisons, the two chicks inoculated with saline only act as valuable reference birds.

TABLE 8. — EXAMPLE OF THE ICPI TEST

State of chicks after inoculation	Day								Total
	1	2	3	4	5	6	7	8	
Normal	10	9	9	6	6	6	6	6	58
Sick	0	1	0	3	0	0	0	0	4
Dead	0	0	1	1	4	4	4	4	18
Total recordings									80

The test is continued for eight days. The values for normal and sick birds are recorded; the total for those dead is cumulative so that a total of 10 recordings is made each day for each group of 10 birds. The observations are weighted so that birds appearing normal are given a zero score, those appearing sick are given a score of 1, and those dead (cumulatively) are given a score of 2.

In the example given in Table 8 the recordings are as follows:

Normal observations	58	weighting	0	total	0
Observations of sick	4	weighting	1	total	4
Observations of dead	18	weighting	2	total	36

The ICPI is expressed as the weighted mean over the number of observations made, namely:

$$\frac{4+36}{80} = 0.5$$

For a velogenic isolate, an example of the ICPI test is given in Table 9.

Intravenous pathogenicity index. This test is similar in design to the ICPI test, but is conducted in six-week-old susceptible chickens. As in the ICPI test, the virus is administered as a 10^{-1} dilution of fresh infected allantoic fluid. In the IVPI test, 0.1 ml of the virus dilution is injected intravenously in each of 10 six-week-old birds.

TABLE 9. — EXAMPLE OF THE ICPI TEST FOR A VELOGENIC ISOLATE

State of chicks after inoculation	Day								Total	Weight	Sum
	1	2	3	4	5	6	7	8			
Normal	10	10	0	0	0	0	0	0	20	0	0
Sick	0	0	3	0	0	0	0	0	3	1	3
Dead	0	0	7	10	10	10	10	10	57	2	114
Total recordings									80		117

Therefore, the ICPI $= \frac{117}{80} = 1.46$.

The inoculated chickens are observed daily, at the time corresponding to the time of inoculation, and the results recorded. For the IVPI test, the birds are recorded as healthy, sick, paralysed or dead. Dead birds are recorded cumulatively. It is advisable to include two control birds which are injected with sterile saline. However, it should be remembered that in the case of virulent contagious virus, the disease will spread from the inoculated birds to the controls, and this spread will be evident clinically at about day eight.

Birds are classified as sick if they huddle together, are disinclined to move, feed or drink, but do not show any marked signs of wing or leg paralysis.

Paralysed birds are those which show clear incoordination of wings or legs, or which are off their feet.

In the IVPI test, a normal bird is weighted zero, a sick bird is weighted 1, a paralysed bird 2, and a dead bird 3 (See Table 10).

The IVPI equals the weighted value over the number of observations made:

$$\frac{262}{100} = 2.62$$

TABLE 10. — EXAMPLE OF THE IVPI TEST WITH VIRULENT VIRUS

State of chickens after inoculation	Day										Total	Weight	Sum
	1	2	3	4	5	6	7	8	9	10			
Normal	10	0	0	0	0	0	0	0	0	0	10	0	0
Sick	0	0	0	0	0	0	0	0	0	0	0	1	0
Paralysed	0	8	0	0	0	0	0	0	0	0	8	2	16
Dead	0	2	10	10	10	10	10	10	10	10	82	3	246
Total recordings											100		262

From the examples given it can be seen that the maximum value of an ICPI test is 2.0 representing all birds dead at the end of 24 hours. Similarly, the maximum value of the IVPI test is 3.0.

The preceding three pathogenicity tests have a reproducibility of approximately ± 10 percent, and should be used for purposes of broad classification rather than for the distinction of viruses of similar virulence.

It is important to appreciate that a low index of virulence does not guarantee strain purity, because it is possible for a lentogenic strain to be contaminated with a more virulent virus without the latter being apparent from the results of the tests. The tests should be used for the classification of laboratory-adapted strains which have been passaged using strict techniques.

Plaque morphology. At present little is known concerning the techniques necessary for the identification of more virulent virus contaminants in the seed virus. The most reliable technique is to seed dilutions of the virus on chicken kidney cell monolayers with an agar overlay and examine the resulting plaque morphology. Virulent virus of the Herts '33 type produces a preponderance of large well-defined plaques after about three days' incubation, while a vaccine virus of the B1 type results in a very poor plaque formation. The latter may only be detected after six days' incubation as small indistinct pinhead plaques. With most isolates the plaque formation is heterogeneous, even after purification by limiting dilution passage. Not all virulent strains exhibit clear plaques, and the isolates from Near Eastern countries and from those associated with outbreaks of the disease in Europe behave in cell culture in a way more similar to mesogenic than velogenic virus.

Examples of pathogenicity tests

TABLE 11. — EXAMPLES OF PATHOGENICITY TESTS ON A SERIES OF ISOLATES

Strain	ICPI	IVPI	MDT
Ulster 2C	0.0	0.0	0.0
Queensland V4	0.16	0.0	0.0
F	0.25	0.0	119.0
B1	0.25	0.0	117.0
La Sota	0.15	0.0	103.0
Komarov	1.41	0.0	69.0
H (Herts)	1.18	0.0	48.0
Roakin	1.45	0.0	68.0
Mukteswar	1.44	0.08	46.4
England '66	1.70	1.70	70.0
Holland '70	1.82	2.53	58.6
England '70	1.86	2.53	60.0
Herts '33	2.00	2.71	48.0
GB Texas	1.75	2.66	55.0
Milano	1.86	2.81	50.0
Asiatic '68	1.96	2.41	49.0

It should not be assumed that the pathogenicity figures in Table 11 give an accurate assessment of the listed strains' usefulness as vaccine challenge strains. For example, two different passage lines of Herts '33 have the following values:

Strain	*ICPI*	*IVPI*	*MDT*
Herts '33/'64	1.94	2.60	—
Herts '33/'56	2.00	2.71	—

While these differences could be within the limits of testing error, it was found that the 1956 line was significantly more lethal than the 1964 line when used as an intramuscular challenge virus in the same group of experimentally immunized birds.

10. THE USE OF NEWCASTLE DISEASE VACCINES

Types of vaccine available

The following types have been produced:

Live lentogenic egg-adapted
Live mesogenic egg-adapted
Live mesogenic tissue culture-adapted
Live vaccine with mineral adjuvant for injection
Inactivated

Live lentogenic vaccines

These are sometimes referred to as vaccines of the B1 type. The best-known strains are the F (Asplin, 1952), B1 (Hitchner and Johnson, 1948), and La Sota (Winterfield *et al.*, 1957).

Individual vaccine production units may produce vaccines which provide an immunity similar to that created by the above strains, but with vaccine virus of different origin.

Generally, the F and B1 vaccines do not cause nervous disease in day-old chicks unless injected intracerebrally, but may give rise to mild and transitory respiratory symptoms. The F strain usually causes the least reaction and the B1 strain generally causes little or no clinical effect. The La Sota strain often causes more post-vaccination respiratory symptoms. The immunogenicity of these three strains is usually considered to be comparable to the degree of reaction induced. The strains grow readily in embryonating eggs to a titre of more than $10^{9.0}$ EID_{50} per ml. When used as immunizing agents, the immune response is largely dependent on the dose of virus administered. This characteristic is more apparent with the milder strains, and generally less with the La Sota strain. For the lentogenic vaccines, the optimum dose is usually considered to be between $10^{6.5}$ and $10^{7.0}$ EID_{50} per bird.

Live mesogenic vaccines

These include the Roakin strain (Beaudette *et al.*, 1949), the Komarov

(or Haifa) strain (Komarov and Goldsmit, 1946), the Hertfordshire (or Herts) strain (Iyer and Dobson, 1940), and the Mukteswar strain (Haddow and Idnani, 1946). For the live mesogenic vaccines, the optimum dose is approximately $10^5 EID_{50}$ per bird, administered by the parenteral route. The mesogenic vaccines are not recommended for the immunization of chickens under 8 weeks of age. Nor are these vaccines recommended for adult birds not previously immunized. The mesogenic vaccines provide a long-lasting immunity.

The Roakin strain was isolated in the United States as a naturally occurring mesogenic strain. It has been used as a booster vaccine.

The Komarov strain was developed by serial intracerebral passage of virulent virus through ducklings. The strain has a mean death time of approximately 70 hours, and is used as a booster vaccine.

The Hertfordshire strain was adapted from virulent virus (Herts '33) by serial passage through eggs. The strain has a mean death time of about 48 hours and can cause reaction in chickens under 8 weeks of age, and in laying hens. It has been used in a number of European countries for boosting immunity. The strain has been combined with fowl pox wing-web vaccine.

The Mukteswar strain was obtained by passage of virulent virus in chick embryos. The strain is the most virulent of the mesogenic vaccine strains and has a mean death time of approximately 46 hours. The intravenous pathogenicity index is 0.08. The strain is used for boosting the immunity of chickens previously vaccinated with a lentogenic virus.

Live tissue culture vaccines

Adaptation of strains of Newcastle disease virus to mammalian cell culture systems has resulted in the production of attenuated vaccines which, although not contagious, are capable of infecting the chicken by inoculation. A vaccine has been attenuated from the strain Cal 11914, and has been adapted to pig kidney monolayer cells. Another vaccine has been produced by the adaptation of the Komarov strain to bovine kidney monolayer cells (Huygelen and Peetermans, 1963). The Mukteswar strain was adapted to pig kidney monolayer cells and used as a mesogenic vaccine in the field (Vasić, 1965). These vaccines are injected into fully susceptible birds and give rise to sustained antibody levels. The results of similar studies have been reported by Markovits and Tóth (1964).

Live vaccines with adjuvants

Vaccines of this type for intramuscular injection have been based on the blending at the time of use of B1 or La Sota vaccine with aluminium

hydroxide gel. The mesogenic H strain was tested after adsorption to aluminium hydroxide gel; its pathogenicity and immunogenicity were lower than those of the H strain vaccine prepared with physiological saline (Palatka and Tóth, 1966).

Inactivated vaccines

These vaccines are made by the inactivation of infected AAF by formalin or BPL (see Chapter 6). They usually have an adjuvant base of aluminium hydroxide gel, or may be in the form of an oil emulsion produced by the emulsification of AAF and a mineral or vegetable oil. Oil-based vaccines have greater volume than live freeze-dried products. Therefore, more space is needed in refrigerated storage and transport.

Choice of types of vaccine

Where the natural disease is mild, an acceptable form of vaccination is the drinking water application of the B1 strain. Usually, vaccination is begun with the B1 strain, and the more virulent and immunogenic La Sota vaccine is used for revaccination. In birds that are mycoplasma-free, it has been found that adverse reactions due to the initial vaccination with the La Sota strain are slight, and in some vaccination programmes this strain is the main vaccine used.

The administration of lentogenic vaccines by the drinking water route does not always produce a response in each individual bird high enough to give effective flock protection. Individual application of the vaccines by the eye drop or intranasal routes results in a more pronounced and more consistent antibody response.

Injected inactivated vaccine stimulates a greater immunity than can be achieved by the use of repeated doses of lentogenic virus administered by the drinking water route. The advantage of inactivated vaccines is that they do not cause stress or induce respiratory disease. Their principal disadvantages are that potent inactivated vaccines are more expensive to produce than live vaccines, and the labour of individual application by injection may make them economically unattractive.

Another vaccination method is to use a lentogenic vaccine as the initial or primary dose, followed by a mesogenic vaccine for revaccination. This programme is used in areas where the virulence of the field virus requires the establishment of a higher and more consistent antibody response than can be achieved by live lentogenic strains.

In summary, the choice of vaccines may be based on equating the degree of immunity necessary, the costs involved and the exposure to local field virus both in terms of its virulence and prevalence. Vaccination

should be kept to a minimum for economic reasons and to reduce the amount of stress and respiratory lesions. The number of applications of vaccine should not be excessive; it has been found that it is more satisfactory to adhere to a tested programme of vaccination than to apply vaccines at frequent intervals. It must be emphasized that the degree of immune response is closely correlated with the concentration of the virus in the vaccine (Tóth and Markovits, 1964). In general the more active vaccines (mesogenic) are used in areas where the field disease is severe. A vaccination programme based on lentogenic vaccines is adopted in areas where the field virus is of lesser virulence.

Since the severe outbreak of Newcastle disease in Europe that started in 1970, many vaccination programmes have been revised to obtain as much protection as possible. Lentogenic vaccines and inactivated vaccines of high quality applied with care can result in degrees of protection that are equal to those obtained with mesogenic strains, and the degree of stress that the latter induce has tended to make them less attractive in intensive poultry units.

Routes of application

The main methods of application of Newcastle disease vaccines are by injection or wing-web stab, eye or nostril drop, drinking water, and spray or aerosol administration.

The first two are individual immunization methods which result in the most uniform dosage and immune response in a flock. Their chief disadvantage is the penning and handling of the birds, which are stressing to poultry and expensive in terms of labour.

The range of immune response in a flock is greater when the vaccine is given by the drinking water route compared with intramuscular inoculation or the eye drop route. Inactivated vaccines tend to give less variation in the immune response than live vaccines. When the immune response to B1 and that to La Sota are compared, the La Sota response, while usually higher, tends to be more variable.

The usual dose of inactivated vaccine is 0.5 ml administered intramuscularly for a chicken or turkey poult. For growing or adult turkeys, the dose should be 1 to 2 ml. The antigenic response following primary vaccination is a direct function of the amount of antigen injected. The secondary immune response is not so dose-dependent; thus the volume of vaccine for turkeys is from 1 to 2 ml per bird.

Most lentogenic vaccines have an affinity for the respiratory epithelium and are more effective when applied individually via the respiratory tract. Thus, the eye drop method of vaccination results in an antibody response which is about four times greater than that attained by vaccination via the

drinking water. In addition, the eye drop method results in a longer duration of immunity and a higher degree of flock protection.

The injectable forms of live vaccines combined with adjuvants require individual administration, and these vaccines are likely to be more useful for small flocks of replacement breeders than for large broiler units.

The tissue culture attenuated vaccines also need to be injected, and their application is generally confined to groups of birds in which individual techniques are more practical.

The mesogenic vaccines are administered either by injection or by wing-web stab. In the latter procedure, usually a mesogenic Newcastle disease vaccine strain and a fowl pox vaccine are given simultaneously. Balla *et al.* (1976b), using the mesogenic H vaccine combined with fowl pox vaccine, have obtained the same level of Newcastle disease immunity in large commercial flocks as that obtained by separate vaccinations. It is important to use a 4-6 branch stick-needle for the combined vaccine.

Should it be necessary to mark the vaccinated birds, a vaccine diluent containing 1 percent *carbo activatus* can be used. Injection into the wing-web will leave a greyish area which persists for about one year.

All mesogenic vaccines, even those which are normally administered only by wing-web stab, have some degree of contagion which increases rapidly on natural passage from bird to bird. While they may be completely safe on single sites, mesogenic vaccines have the disadvantage of possibly spreading to younger unprotected birds and causing a variable degree of respiratory reaction.

Drinking water. In large flocks in excess of 10 000 birds, especially in battery systems, individual application becomes impracticable and reliance has to be placed on mass methods of administration, the most common of which is application via the drinking water. This route may give a minimum response and is generally the least effective way of vaccination. However, reports indicate that water vaccination can give satisfactory results (Owolodun and Ajiboye, 1975). In some flocks during the last severe Newcastle disease epizootic (1970-73), vaccination by the drinking water route resulted in a poor or uneven distribution of the immune response. This applied especially to battery systems, where technical faults may have occurred with long tube systems, low water pressure, and the nipple-drinker system (Dorn, 1974).

As the drinking water method is still the most common means of Newcastle disease vaccination, attention should be paid to conducting the vaccination strictly according to the directions (Spalatin and Hanson, 1974).

When vaccine is applied via the drinking water, care must be taken to ensure that the drinkers and any piped water system that is used to convey

the vaccine are clean and free from detergents, disinfectants or debris that may inactivate or adsorb the vaccinal virus.

Gramenzi (1964) has reported that small amounts of chlorine, iron, zinc or copper can cause a marked reduction in the concentration of viable virus, but Aller and Allan (1969) did not find that galvanized zinc containers caused rapid loss of infectivity, and Allan (1973) found that copper levels up to 1 part per million did not cause appreciable inactivation. The addition of 1 part skim milk to 400 parts of vaccine containing water will generally give effective protection to the virus (Gentry and Braune, 1972; Michaelis, 1975). Water containing a high level of free chlorine should not be used. In areas where water is frequently dirty or contaminated, the use of distilled water is advisable.

In the case of birds on free range, the vaccine solution should not be exposed to sunlight.

Administration by the drinking water route is affected by the speed at which all the birds drink. It is customary to deprive birds of water for about two to four hours before placing the vaccine-containing water in front of them, and it is essential that there be enough drinker space for all birds to drink evenly. The vaccine should be diluted according to the age of the birds so that an adequate amount of vaccine is allowed. The quantity of water generally required per bird for the drinking water vaccination is as follows:

for 10- to 14-day-old birds	10-15 ml
for 3- to 8-week-old birds	20-30 ml
for older birds	40 ml

Spray or aerosol. This mass administration technique involves the application of virus in the form of a spray or aerosol, the former having an initial droplet size of from 10 to 100 microns and the latter from < 1 to 50 microns. Narvaez (1976) reported that in aerosol vaccination over 90 percent of the particles emitted were between 0.5 and 3.0 microns in size.

In general, aerosol vaccination techniques give the most reliable immune response, and can be used effectively in closed houses to give emergency boosts to immune levels in the face of local challenge.

The better immune response of birds to the spray or aerosol vaccination may be explained by two basic facts:

— Airborne infection is one of the most important natural routes of Newcastle disease virus infection.
— The respiratory epithelium is very susceptible to Newcastle disease virus. Often the virus multiplies in the respiratory epithelial cells to higher titres than in other tissues.

A better local antibody production in the respiratory tract in aerosol-immunized birds was demonstrated by Fleischer (1974). Other reports have considered the high and even immune response when susceptible flocks are vaccinated using the aerosol method (Allan, 1975; Eidson and Kleven, 1976; Balla *et al.*, 1976a; Pieper, 1974; Graunke, 1975).

When Newcastle disease vaccine is given by the aerosol route under well-defined conditions, a higher and more even response results. This response is measured by individual HI titres of vaccinated birds and their ability to withstand challenge. The aerosol route is particularly effective when used to vaccinate chickens that have maternally acquired passive immunity; however, this route may result in severe reactions and an increase in air sacculitis. Therefore, the tendency is to discontinue aerosol vaccination in periods of low disease risk.

The particles of water-based vaccine that are generated by an aerosol machine are subject to desiccation in under a second, unless the relative humidity is very high. As the particles approach dryness, there is considerable shrinkage in size and the concentration of dissolved salts in the droplets rises rapidly. For this reason, tap water, which contains various dissolved salts, may produce droplets which have high salt concentrations when they dry out. These conditions can inactivate the vaccine virus. The addition of small amounts of defatted dried milk at 1:1 000 or gelatin gives added size to the droplets and acts as a protein protectant. This procedure has been found to result in the greatest recovery levels of live vaccine virus from the air of the poultry house in which the aerosol has been released and from birds immediately after they had inhaled the aerosol (Gough and Allan, 1973, 1976).

The bulking effect of various concentrations of dry matter (dried milk) on the dry particle size in relation to the initial wet particle diameters has been examined. Initial particles of 10-micron diameter with 1 percent milk powder produced nuclei with a radius of 1.07 microns, those with an initial diameter of 30 microns resulted in nuclei of 3.23 microns, and 50-micron particles resulted in nuclei of 5.39 microns. As the dry matter content and initial diameter rose, the resulting nuclei increased in size.

Most commercial aerosol generators produce a wide variety of droplet sizes. The larger droplets account for a greater part of the original volume of vaccine, and this may be largely wasted. The smaller and more penetrative particles use less vaccine liquid and usually occur in large numbers.

In practice, commercial vaccination is carried out with initial particles of 50 microns with 0.1 percent dried milk additive. This gives final particles of 5 microns.

Vaccine strains used in aerosol vaccination. It is generally accepted that the Hitchner B1 strain causes less stress than the La Sota strain, and many

operators have used it for the first aerosol vaccination and the La Sota strain for the second and subsequent vaccinations. In Appendix 10 a table of comparative results of different La Sota seed strains demonstrates that there is a detectable variation between the seed strains in use by different companies. Therefore, the degree of reaction may vary with the source of the vaccine. In the past, the protective ability of Newcastle disease vaccines was considered to be associated with the degree of reaction induced. It is now recognized that this is not necessarily the case, and effective vaccines with minimal stress reactions are available.

Dose of virus. Most control authorities now require that the vaccine contain not less than $10^{6.0}EID_{50}$ of virus per field dose for each bird.

Aerosol administration. In aerosol vaccination, a known amount of virus is discharged into a fixed air space rather than being administered directly to the birds. For 20 broilers occupying 1 square metre of floor space, the average volume of air is 0.14 m^3 or less per bird, depending upon the height of the ceiling. For laying or breeding birds the air volume is larger. Also, in tropical and subtropical climates the space allowance per bird is substantially greater. This increased space per bird is not known to have an adverse effect on aerosol vaccination, provided the chicken house is environmentally controlled. The latter consists of a house that is totally enclosed and has the ventilation controlled by fans that can be switched off to provide still air conditions (at low outside wind speeds).

Difficulties may occur in houses with open sides, where a generated aerosol is free to diffuse rapidly out of the building. However, during days when air conditions are calm, a high proportion of birds may be effectively immunized, although the flock response is not as satisfactory as in a controlled environment house. On days when there is a ground wind speed of 8 km per hour or more, aerosol vaccination in open-sided houses is impossible.

In some regions, plastic side curtains have been fixed to the walls of houses to render them more air-tight. This procedure has resulted in a more effective vaccination, although care must be taken to open the ventilation within 15 minutes to avoid suffocation or heat stroke occurring in the flock.

Volume of water per dose. The speed of the operation within the poultry house and the speed with which the aerosol machine generates the aerosol determine the amount of water needed. In fixed-flow machines the volume of water required must be determined by trial and error before the vaccine is added. In machines with an adjustable setting, a flow rate of 100 ml per 3 minutes per 10 000 birds generally gives a comfortable working speed.

This flow rate generally produces smaller and more penetrating aerosol particles. The more penetrating particles result in an increased immune response.

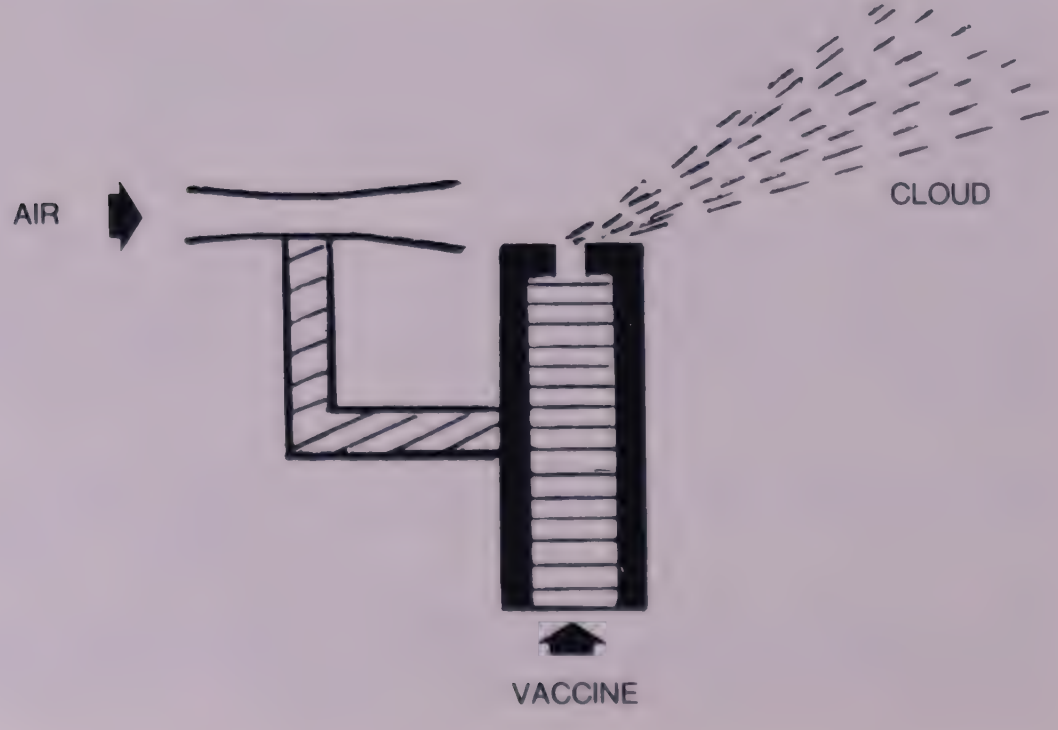
Significantly greater flow rates should be avoided because this causes the relative humidity in the house to rise to unnecessarily high levels. This high humidity results in a high proportion of very large aerosol droplets, which rapidly fall to the ground and are not inhaled.

Quality of water. As an aerosol particle dries, the salt concentration increases and finally the dry particle comprises only the carbonates, other salts and suspended solids, and the vaccine virus. These changes are marked when tap water is used, and cause a serious drop in virus titre with a resulting failure in immunization.

When high-quality distilled and deionized water is used, the evaporation will result in the final smaller droplet containing only the virus particles and the suspending agents. A suspension containing dry matter at 1:1 000 will give rise to dry droplets one tenth of their initial diameter. Thus the composition of the suspending fluid and the initial range of droplet sizes has an important bearing on the final aerosol particle size.

Aerosol generators. There are two basic types of aerosol generators in current use. One type uses the venturi effect (Figure 15) for droplet

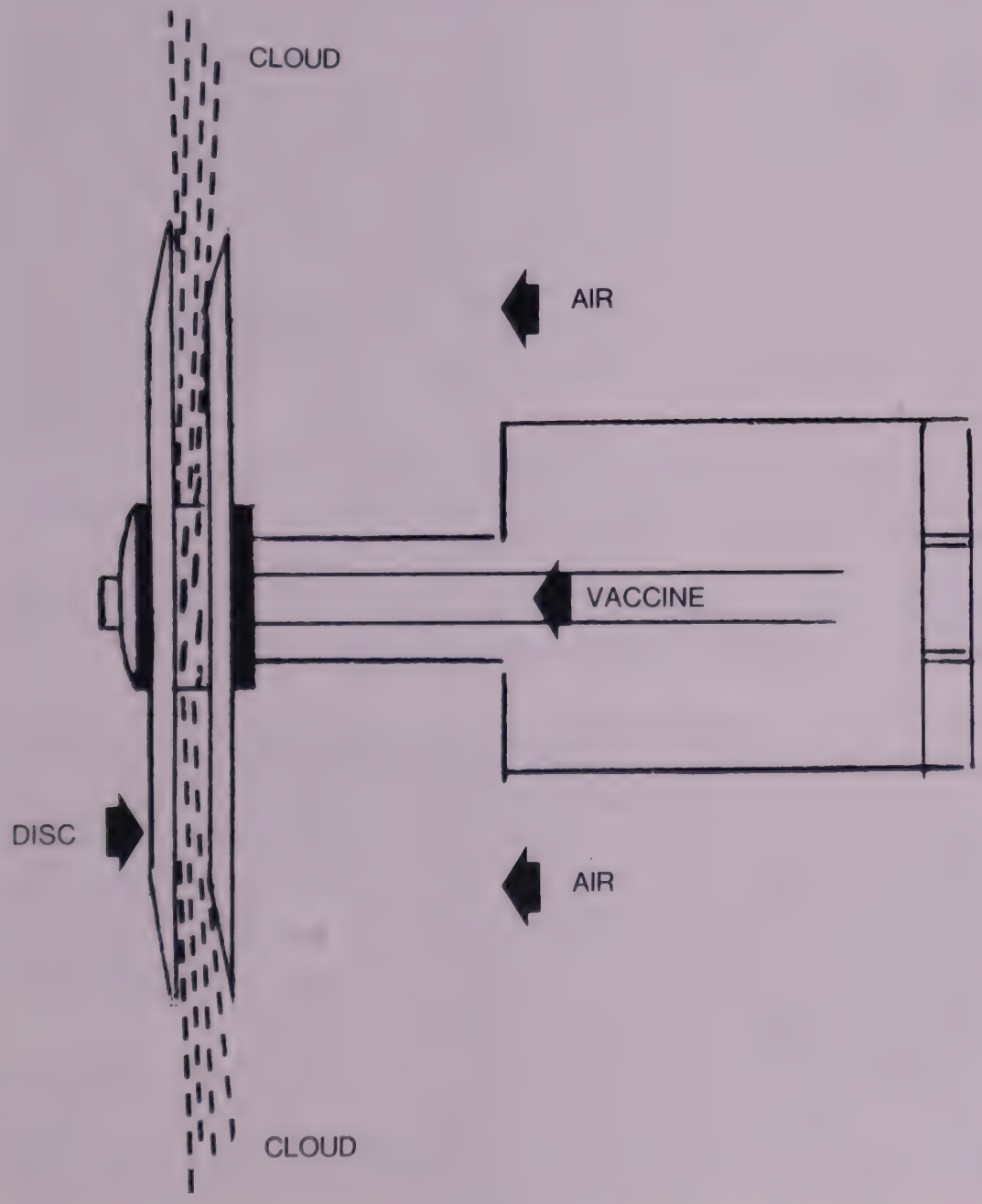
FIGURE 15. The venturi principle for aerosol generators



production. In the second type, the droplets are spun off a spinning disc (Figure 16).

In some machines, the flow of vaccine to the nebulizer nozzle is by gravity; in other machines, the flow of vaccine depends on the pressure generated by the blower unit. Some generators have no flow adjustment, while others have a screw setting to allow a slow or fast liquid flow. A slow setting tends to give fine particles and use relatively little diluent in unit

FIGURE 16. The spinning disc principle for aerosol generators



time, while faster flow settings produce larger droplets and use more diluent.

In theory, the spinning disc type of machine should provide droplets of a constant size, but particle analysis of resulting clouds has shown that the particle range of the spinning disc type of generator is almost as wide as that of the venturi machine. This result may be due to the fact that the metal of the discs is not wettable, and as a result an even film of liquid is not produced.

In the machines tested, most particles had a range of 40 to 60 microns in initial diameter, which in suspensions of 1:1 000 dry matter gave particles of 4 to 6 microns (Gough and Allan, 1973).

When the orifice of a generator is large, or is set to give maximum flow rate, a proportion of particles will have diameters in excess of 200 microns. These particles are clearly visible, fall quickly to the ground and represent lost vaccine. When there is a small number of large particles, the proportion of vaccine in these particles may be very large. Therefore, no machine should be used that produces this " spitting " effect. The majority of machines are electrically driven and must be fitted with long lengths of flex to allow the operator to move freely in the poultry house. Most generators are fitted with a powerful fan which discharges the aerosol in a wide cone for a distance of approximately 9 metres. Machines not fitted with a powerful fan project the aerosol less than 2 metres. Unpublished results indicate that the " Vulcan," " Microsol " and " Tubair " machines have a long throw, while the " Atomist " machine has a short throw. Prior to purchase, the manufacturers should provide sufficient information regarding the operation of the generating machine.

Some machines are very noisy, and nervous birds may panic and smother. In this situation, operators should first accustom the birds to the noise of the aerosol machine operating outside the house.

Safety to man. It should be remembered that the operator also inhales the aerosol unless he wears a well-fitting mask with an extremely fine filter. The effect of a Newcastle disease vaccine aerosol on man is usually most marked on the first exposure, when conjuctivitis and asthma-like symptoms develop. Most operators report that the effects soon disappear after repeated aerosol exposure. However, it is strongly advised that operators wear an effective mask, and that people with a predisposition to asthma not be allowed to apply virus aerosols or be present in the building during or immediately after the aerosol has been produced.

In addition to the virus itself, the vaccines also contain egg material from the allantoic fluid, and most vaccines also contain some milk, peptone and a number of sugars. The protein constituents are capable of inducing asthmatic reactions in man. For anyone seriously affected, med-

ical aid should be obtained immediately.

Vaccination programmes involving aerosol application. Aerosol vaccination may be part of a routine preventive programme in which good immunity is required with a minimum of reaction, or vaccination may be needed to suppress an outbreak of Newcastle disease.

On farms where the parent birds have been vaccinated but not infected with field virus, the maternal immune levels of Newcastle disease antibody in the progeny chicks may be expected to be at a low level at 2 weeks of age. Thus it is best to give the first application of aerosol vaccine shortly before the disappearance of maternal antibody by vaccinating the chicks with the B1 strain at approximately 10 days of age. The immunity so conferred will tend to protect the birds from the side effects of a subsequent aerosol exposure given within a short period of time. Therefore, the La Sota vaccine may be given as the second dose at 20 days of age and the third at 30 days of age. This intensive vaccination would only be applicable in periods of high disease risk. The application of an intensive vaccination programme together with adequate disinfection measures may result in freeing successive crops of broilers from infection.

During periods of lesser risk, a single dose of La Sota vaccine at 21 days of age will provide good immunity in a broiler flock, although the immunity level may be variable.

When developing a vaccination programme for a specific region or farm, it is essential that blood samples for HI testing be taken to establish the effect of the response to the vaccine used. The response may be affected by unusually high levels of maternal antibody due to infection in the parent flock, or by the immunosuppressive effects of Gumboro disease (infectious bursal disease), which may cause the Newcastle disease vaccination to be of little value (Allan *et al.*, 1972; Faragher *et al.*, 1974; Pattison and Allan, 1974).

During a serious outbreak of Newcastle disease on a large site or in an area with a dense poultry population, aerosol vaccination may be used as a " fire-break " method or " ring vaccination " to stop the spread of disease (Hutchinson, 1975). The reason that aerosol vaccination can be used in this way is that the immune response commences at two and a half days (Gough and Alexander, 1973), while the immune response to other forms of vaccination may require up to 10 days to develop.

To control a disease outbreak, La Sota vaccine should be used with 1:1 000 defatted dried milk in distilled deionized water and should be discharged from the aerosol generator at a slow rate of not more than 100 ml per 3 minutes as a small particle-size aerosol. Vaccination should commence in a non-affected flock far from the disease outbreak and move toward the affected flock or premises. Generally, birds in the affected

house will show a mortality rate unaffected by the emergency vaccination. However, birds near the end of the four-to-six-day incubation period will show a reduced mortality following vaccination. In other vaccinated flocks, mortality may be absent.

Adverse effects following aerosol vaccination. It is known that poultry stock infected with *Mycoplasma gallisepticum* are more susceptible to respiratory reactions following aerosol vaccination, although mycoplasma eradication has helped minimize the vaccination reaction. Other adverse effects may be due to infection with *Mycoplasma synoviae* (Villegas *et al.*, 1976), coli-septicaemia (Gross, 1961) or an unknown cause. Generally, poultry veterinarians with experience of the flock can judge the clinical reaction and level of immunity likely to result from a particular route of vaccine administration.

11. VACCINATION PROGRAMMES

The requirements of immunization vary with local circumstances, and no overall or specific programme of vaccination can be defined (Lancaster, 1964). In selecting the most effective and practical programmes, consideration must be given to the levels of protection needed, the immune status of the birds, the type of field virus occurring in the locality, the relationship between other poultry diseases, the administration of the Newcastle disease vaccines and the monitoring of the immune response.

The present general view is that successful vaccination against the disease is dependent on the amount of antigen which enters the tissues of the bird. This in turn depends on the successful application of the vaccine. There is no evidence to indicate the need to relate a vaccine to the antigenic type of field virus occurring in any one locality (Chu and Rizk, 1971).

The range of immunity within a flock may vary considerably. There is a difference between disease resistance and infection resistance (Beard and Easterday, 1967). The former relates to resistance to overt or clinically apparent infection. The latter implies a reduction in viral replication and spread within the host tissues. Individual birds with low levels of immunity may survive exposure to virulent field virus, but continue to harbour the field virus as a latent infection. Thus, Utterback and Schwartz (1973) reported that during the 1971-73 Newcastle disease epizootic in California, United States, vaccination reduced mortality, but did not prevent a flock from becoming infected with the VVND virus. Vaccinated flocks maintained infection with field virus and shed virus for varying periods up to four months. Therefore, if the flock is likely to be exposed to virulent field virus, it is very important to raise the immunity level as high as possible.

Genetic effects

Peleg *et al.* (1976) found that the genetic constitution of the birds may have a significant effect on the response to vaccination. These authors suggested that selection for response to inactivated Newcastle disease vaccines might improve the response to live attenuated vaccines.

Transmissibility of vaccine strains

Spalatin *et al.* (1976) showed that the La Sota strain produced full immunity in birds in contact with vaccinated birds. However, the degree of vaccine virus spread is likely affected by a number of variables. Thus, Belić *et al.* (1968) reported that following vaccination with the Mukteswar virus, this virus could be recovered from the cloaca 12 days after vaccination.

Factors affecting the immune response

Susceptible chicks are able to respond to vaccination at an early age and generally reach full immunological maturity at about 10 weeks of age. In the application of vaccination, the first consideration is the immune status of the chicks. The parent flocks may have antibody to Newcastle disease virus as a result of vaccination or exposure to field infection. Also, the amount of Newcastle disease antibody will vary considerably from flock to flock and between individual birds in the same flock. The antibody is passed to the yolk, and thence to the developing chick during the later stages of incubation. The yolk sac is fully absorbed within a few days after hatching. The level of passively acquired antibody in the serum of day-old chicks is approximately the same as in the serum of the dam.

The level of passively acquired maternal antibody in the young chick generally declines at a constant rate (Figure 20) and has a half-life of approximately four and a half days. This means that every four and a half days, the level of antibody falls by half. Thus, using the HI test, a day-old chick having an HI titre of 2^7 can be expected to have an HI value of 2^6 at 4½ days, 2^5 at 9 days, 2^4 at 13½ days and 2^3 at 18 days, at which time the chick will respond to vaccination. If a live virus vaccine is administered to a congenitally immune chick under 18 days of age, the antibody will inhibit the multiplication of the virus. Antigen inoculated in the form of inactivated vaccines may also be neutralized. It is considered that the administration of a vaccine to a chick in the presence of significant amounts of circulating maternally derived antibody increases the rate of removal of antibody, and renders the chick more responsive to subsequent application of vaccine (Quaglio and Lombardi, 1973).

When parent flocks have been infected with virulent virus, significant amounts of antibody can persist for 42 days in individual chicks. This antibody has not protected chicks against infection with virulent virus (Karczewski, 1973). The levels of passive HI antibody in groups of chicks from infected parent flocks have been found to vary from less than 2^3 to greater than 2^{15}. This variation in level of antibody is found when hatcheries obtain hatching eggs from parent flocks which may have been

recently infected or may have low antibody levels resulting from a previous vaccination. The effective vaccination of a flock of chicks having a varying passive immune status can be very difficult (Gangopadhyay and Mallick, 1971). The existence of a proportion of birds with low levels of passive antibody results in some of the flock being susceptible to field infection at an early age. These chicks are capable of maintaining field infection in the flock, and as the level of passive antibody declines in other chicks, these in turn become infected and perpetuate the field virus. The death rate in such a flock is usually more prolonged than in flocks with a more uniform antibody status. Generally, the peak mortality occurs in chicks 18 to 25 days of age. While vaccination at 1 day of age may give active protection to chicks with the lowest levels of antibody, vaccination at this age may be largely ineffective in the majority of chicks and hence should be regarded as a preliminary step rather than a recognized protective procedure. However, in certain situations La Sota vaccine as a spray has overcome the maternal antibody status of day-old chicks (Pieper, 1974). In flocks of chicks having high levels of maternal antibody, it has been found that approximately 60 percent of the chicks will actively respond to vaccination at 18 to 21 days of age, while at 14 days of age the percentage of chicks that will respond may be under 40 percent. For this reason, it is considered more effective to vaccinate at about 3 weeks of age rather than at 2 weeks, regardless of whether the chicks were vaccinated in the first few days of life. Turkey poults with maternally derived antibody have responded poorly to vaccination within the first 4 weeks of life (Box *et al.*, 1976b).

In flocks with high maternal antibody levels, vaccination at 3 weeks of age gives protection only to a proportion of the chicks. Revaccination at 35 to 42 days of age is required to give the high levels of immunity necessary in areas where the disease is prevalent. The results of revaccination also are affected by the antibody level at the time of vaccination. Where the majority of birds have HI levels of 2^6 or greater, the response to repeated applications of lentogenic live vaccines is poor. In these circumstances, it has been found that revaccination with a potent inactivated vaccine incorporating an adjuvant will give a more marked antibody response. On the other hand, application of a live lentogenic vaccine is more dependent on circulating antibody, and the immune response may be relatively ineffective if revaccination is carried out too soon after a previous vaccination.

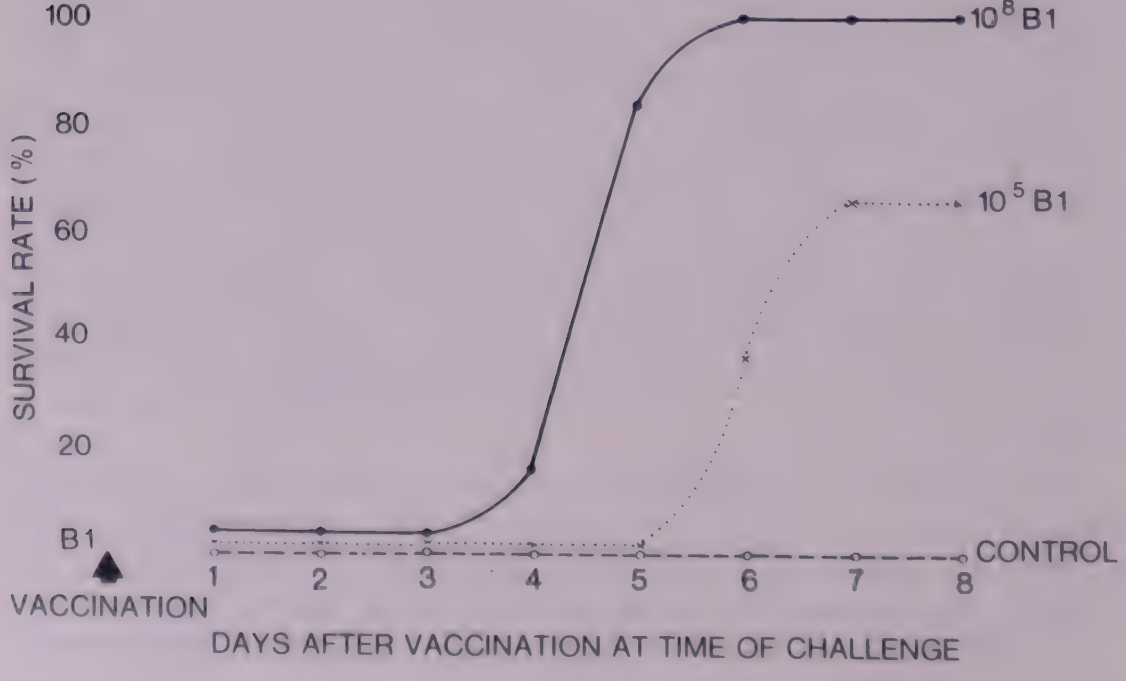
The application of lentogenic vaccines in the presence of infectious bronchitis virus has been shown to adversely affect response (Hanson and Alberts, 1959; Bracewell *et al.*, 1972). The effect is marginal when both vaccines are administered simultaneously. The reduction in immunity is more marked if the Newcastle disease vaccine is given on the tenth day

following the infectious bronchitis vaccination. For this reason, where the response to the Newcastle disease vaccine is important, this vaccine should be given alone (Thornton and Muskett, 1975) or in conjunction with the infectious bronchitis vaccine (Bengelsdorff, 1972), and not during the 14-day period following infectious bronchitis vaccination (Newcastle disease control, 1971).

Vaccination with live Marek's disease vaccine at 1 day of age is a common practice. At present, there is no evidence of an interference between Marek's disease virus and killed Newcastle disease vaccine (Box and Furminger, 1971; Box *et al.*, 1976a). However, Marek's disease vaccination has slightly reduced the immunity to Newcastle disease following a live Newcastle disease vaccine administered at 1 day of age (Picault *et al.*, 1974). Other authors have not reported this effect (Meulemans *et al.*, 1974).

Infectious bursal disease (Gumboro disease or infectious bursitis) is widespread in many countries, and it has been shown that exposure to this disease early in life can have a marked adverse effect on the ability of the chick to respond to Newcastle disease vaccination and to withstand subsequent challenge (Allan *et al.*, 1972; Giambrone *et al.*, 1976). However, other studies have shown that prior vaccination against infectious bursal disease did not reduce Newcastle disease immunity (Zanella *et al.*, 1976, 1977; Lombardi, 1974).

FIGURE 17. Development of immunity in B1-vaccinated day-old chicks



Babkin and Pilipchuk (1975) reported that infectious laryngotracheitis delayed the immune response to a lentogenic Newcastle disease vaccine.

Monitoring the immune response

Successful control of Newcastle disease also involves the determination of the immune response produced by vaccination. Siegmann *et al.* (1973) found the HI test to be most useful for this purpose, and suggested that 25 to 30 serum samples be examined two to three weeks after vaccination.

The prevalence of serologically positive birds with HI titres of 1:8 or higher indicated an immune flock (Balla *et al.*, 1975).

The HI test may be conducted with untreated egg yolk (Dorn *et al.*, 1973; Valadão, 1975) and a national system of determining flock immunity has been reported (Roepke, 1973). However, serum titres have risen faster and reached a peak five days before yolk titres (Seidl, 1975).

Levels of protection required

Excessive vaccination is costly and can lead to the development of diseases of the respiratory system. For this reason, vaccination against Newcastle disease should be reduced to the minimum consistent with an acceptable degree of immunity. Concurrent vaccination has resulted in a satisfactory immune response and has overcome the effects of maternally derived antibody. In this procedure, chicks 1 or 2 days old are vaccinated at the same time with an oil emulsion vaccine, together with a dose of live B1 vaccine virus (Box *et al.*, 1976a; Warden *et al.*, 1975; Pagnini *et al.*, 1976).

The effect of natural Newcastle disease in vaccinated birds varies with the virulence of the field virus, the concentration of field virus within an infected house, and the level of flock immunity. Thus, in areas where the naturally occurring field virus is relatively mild (field strains having ICPI values of less than 1.7 and IVPI values of less than 2.0), administration of the B1 type of vaccine in the drinking water may give levels of protection which prevent the natural disease. Where the natural infection is more severe, higher antibody levels are required.

Figure 17 shows the effects of intranasal vaccination in groups of day-old chicks with either 10^8 or 10^5 B1 vaccine per bird dose. The former engendered protection two days earlier than the 10^5 bird dose, and the protection level was also much higher with the 10^8 bird dose than with the 10^5 dose. The results of challenge with virulent virus emphasized the importance of high virus content in the vaccine.

Higher antibody levels may be obtained by individual application of lentogenic vaccines by the ocular or nasal routes, or by the aerosol method of application. Increased protection may also be obtained by the use of potent inactivated vaccines or by the application of mesogenic vaccines (Figure 18).

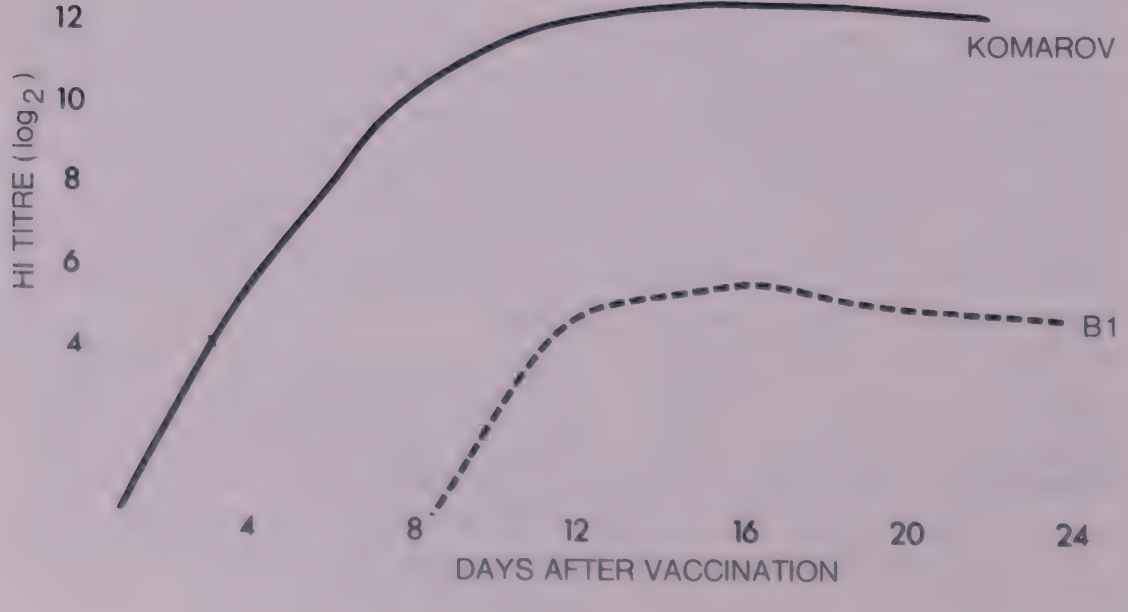
The rate of development of antibody, and hence protection, is important when vaccines are used when there is the threat of disease in an area. Figure 18 illustrates that the mesogenic Komarov vaccine gave rise to antibody levels which were detectable on the third day after vaccination in contrast to the B1 strain where antibodies did not appear until eight or nine days after vaccination.

An early resistance develops in birds inoculated with mesogenic vaccines; 24 to 48 hours after vaccination the birds are resistant to virulent natural or artificial infections. This phenomenon can be explained by virus interference, and is of practical importance in disease areas (Buzna and Hodosy, 1951). Others have also detected a serological response two days after immunizing chicks with the F strain (Higgins, 1971).

Suggested programmes of vaccination

The following programmes are not intended to be recommendations applicable in all circumstances, but rather to illustrate programmes that have been effective under known situations.

FIGURE 18. Rate of HI rise following primary vaccination



When Newcastle disease is mild, sporadic and of low incidence

Vaccination. B1 vaccine by the ocular or intranasal routes or by aerosol application at 1 day of age or at 4 to 5 days of age. B1 vaccine by the drinking water route at 18 to 21 days of age, or La Sota in the drinking water at the same age provided the birds are mycoplasma-free.

Revaccination. La Sota vaccine in the drinking water or by ocular application at approximately 10 weeks of age.

Point-of-lay vaccination. La Sota vaccine by the drinking water route, or inactivated vaccine. In small flocks the individual application of lentogenic vaccine by the ocular or nasal routes is more effective if labour facilities permit individual handling.

Vaccination during the laying period. La Sota vaccine may induce a temporary drop in egg production, and for this reason revaccination is mainly confined to the B1 strain. The latter is repeated usually at three-month intervals by drinking water application.

When Newcastle disease is more severe and more prevalent

1. Application at 1 day of age or during the first five days of life, as above. Or the first aerosol vaccination may be conducted at 10 days of age (see Chapter 10).
2. Revaccination at 21 days of age and again at 35 to 42 days of age. Aerosol vaccination may be used instead of drinking water application.

FIGURE 19. Example of a satisfactory vaccination programme

3. Revaccination at 10 weeks of age with mesogenic or inactivated vaccines.
4. Revaccination at point of lay with mesogenic or inactivated vaccines.

Summary. In a vaccination programme, the following should be considered:

— The pathogenicity of the vaccine virus.
— The immune competence of the chicks.
— The levels of circulating maternally derived antibody.
— The levels of residual antibody from a previous dose of antigen.
— The effect of immunization on the general health of the bird.
— Vaccination programmes for other diseases.
— The general level of immunity required.

Age at vaccination. The B1 and F strains are considered suitable for immunization during the first few days of life. The La Sota strain is not usually given as the initial immunizing agent due to its slightly greater pathogenicity.

If the disease risk to very young chicks or poultry is considered low, it is often best to defer vaccination until the effect of maternal antibody has waned. If the disease risk is serious, early vaccination may be essential, although the majority of birds will not respond to the antigen.

The choice of age for vaccinating young chicks is affected by the levels of antibody received passively from their dams. In Figure 20, the decay rate of antibody from two flocks is compared: in flock I, the parent birds were vaccinated but not infected, and it can be seen that their progeny could respond to vaccination from 14 days of age onward. Flock II had recently been infected with virulent virus; in this flock, levels of antibody known to interfere with vaccination were detectable until 42 days of age. In situations of this kind, approximately 60 percent of the birds will respond at about 21 days, while the remaining 40 percent are refractory until approximately 42 days.

Figure 20 also shows the wide range of titres occurring between two flocks; the chicks with values of 3 can be expected to respond to vaccination from 1 day of age onward. By applying the data in Figure 20 to Table 12, the age at which the birds will respond satisfactorily to Newcastle disease antigen can be estimated.

From the table it will be seen that when the day-old HI values are greater than 7, there is not likely to be an active immune response to vaccination before the chicks are 21 days of age. If the majority of the chicks have HI values of 5 or less at 1 day of age, immunization at 14 days of age can be expected to be successful. In general, the progeny of a breeding stock that

TABLE 12. — AGE OF EARLIEST RESPONSE TO VACCINATION AT VARIOUS DAY-OLD HI TITRES

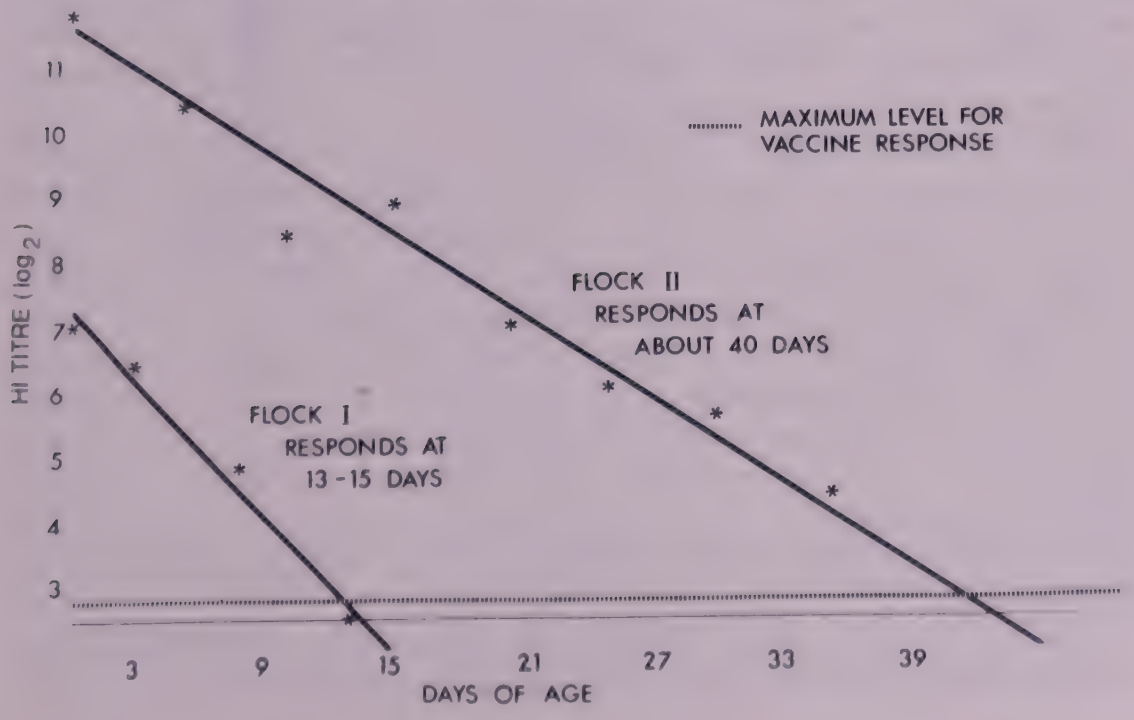
HI day-old value	Earliest age for vaccine responses
..... log_2	..... *Days* .....
3	1
5	9
7	18
9	27

has been vaccinated but not infected with field virus may be expected to be largely free of maternal antibody by 14 to 21 days of age. Therefore, there is a strong case for standardizing the age of vaccination at 14 to 21 days, especially where the incidence of Newcastle disease is low.

The time required for an immune response to the B1 type of vaccine is about six to eight days, and vaccination at 14 to 21 days of age will therefore provide active immunity at 21 to 30 days.

Unless the local disease risk is unusually high, vaccination prior to 14 to

FIGURE 20. Age of response to vaccination with variation in maternal HI levels



21 days of age is likely to be of no value. For the progeny of breeding stock known to have low or nil antibody levels, vaccination may be carried out as desired. In the case of progeny that include a proportion of birds that have HI titres in excess of 7 at 1 day of age, vaccination at 21 days of age will not give complete flock protection. In this case, the vaccination programme should include a further vaccination at 35 to 42 days of age in order to immunize those chicks which have an initially high level of passive antibody.

Revaccination. In the case of broilers in areas where the prevalence of the disease is low, and where the pathogenicity of the field virus is also low, successful vaccination at 14 to 21 days of age will prove adequate, provided that approximately 100 percent of the birds show an immune response. The degree of protection resulting from the drinking water route of application of the B1 type vaccine is less than the protection obtained by ocular or intranasal application.

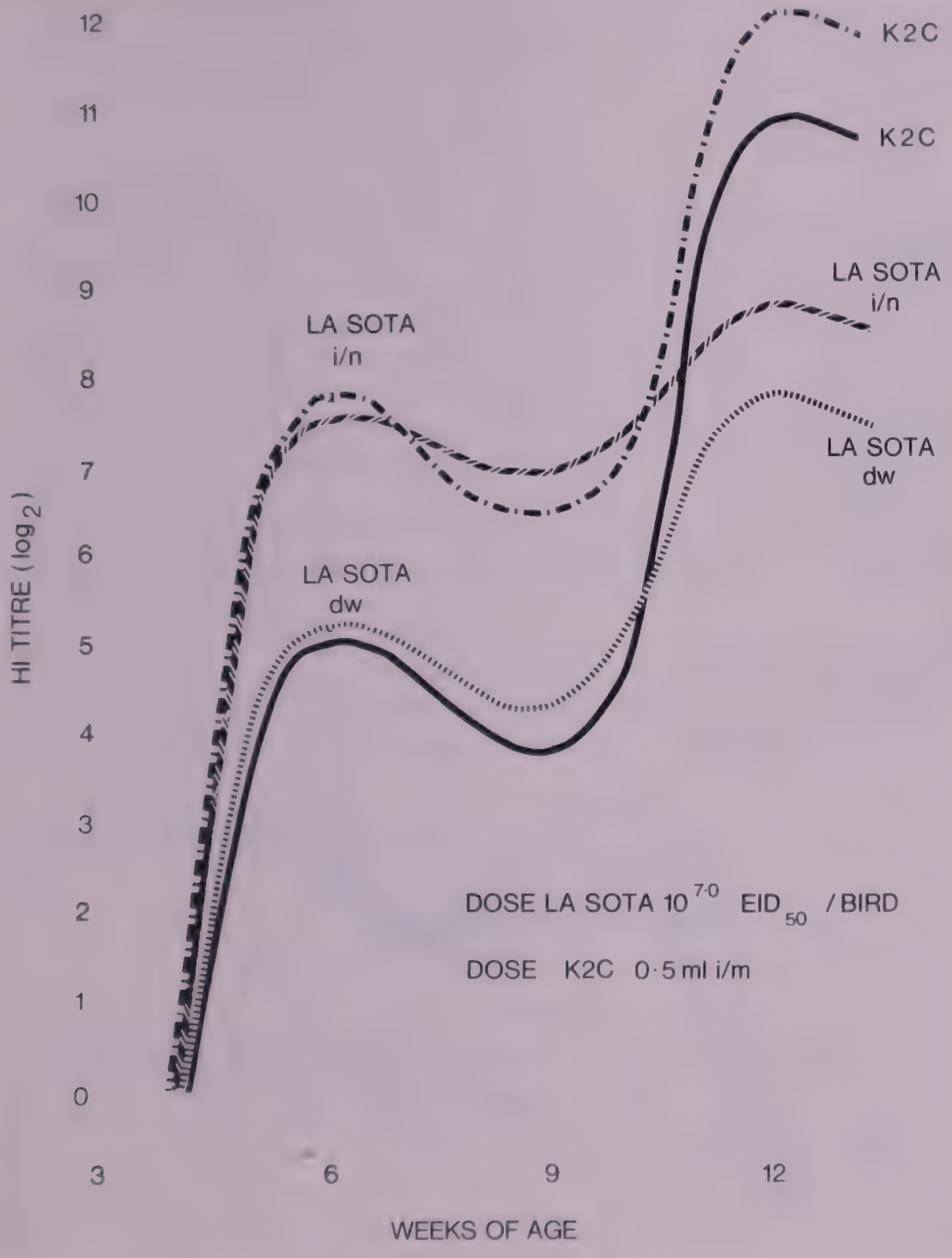
In areas in which the disease risk is greater, it will be found that a single application of vaccine at 14 to 21 days of age may be inadequate. Therefore, it may be necessary to revaccinate in the period under 10 weeks of age. In this case, revaccination at 42 days using the La Sota strain by the drinking water route will provide a satisfactory boost to immunity. If the time interval between the primary and secondary vaccination is less than 21 days, the antibody produced by the first dose of vaccine is more likely to interfere with the multiplication of the second dose of virus. Therefore, there is little to be gained by reducing the interval between vaccinations. As the response to the first vaccination declines with time, delaying the second dose can be expected to elicit a better response. However, in some cases increasing the time between vaccinations increases the risk of losses if exposure to Newcastle disease occurs.

Figures 21 and 22 show the magnitude of the secondary response in groups of susceptible birds immunized at 3 weeks of age and re-immunized at 9 weeks. The data presented are not intended to be the basis of recommendations for a programme of vaccination, but to illustrate the relative immunogenicity of various vaccines and modes of application.

It will be seen from Figure 22 that the response to revaccination with the B1 or F strain by the drinking water route does not lead to an increase in the secondary response over the levels obtained following the primary response. In contrast, the secondary response obtained by revaccination with La Sota vaccine in the drinking water, as shown in Figure 21, is similar to revaccination by individual application using the intranasal or ocular routes.

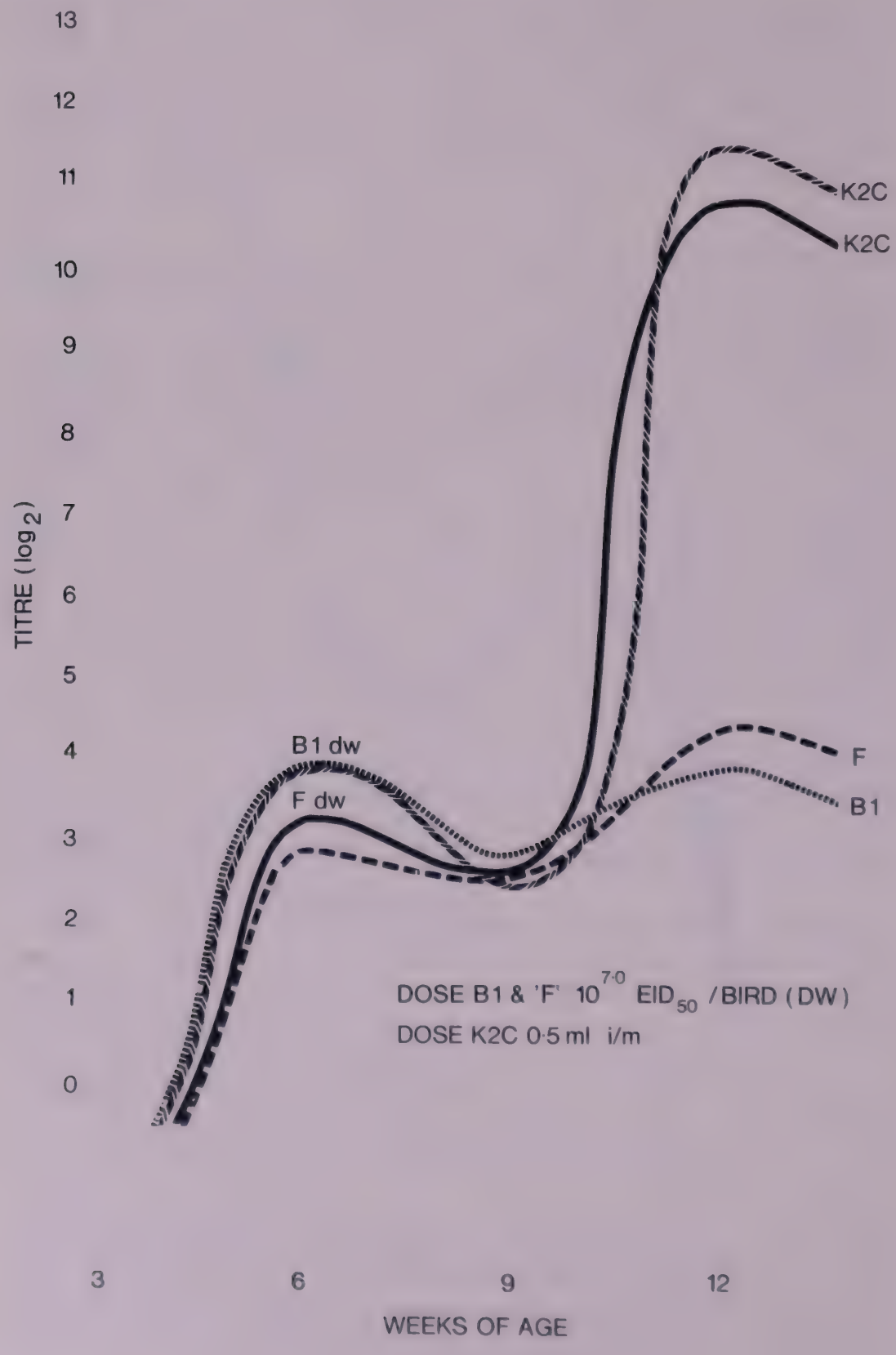
Figures 21 and 22 illustrate the comparative effects of lentogenic live vaccines and inactivated vaccines as primary and booster doses. It can be

FIGURE 21. Vaccination response to La Sota live virus and K2C inactivated vaccine



seen that the milder B1 and F strains produced a significantly lower primary response than the more active La Sota strain. From Figure 21 it can be seen that the response was markedly affected by the route of application of the vaccine. The intranasal route (i/n) gave a significantly

FIGURE 22. Vaccination response to B1 and F live virus and K2C inactivated vaccine



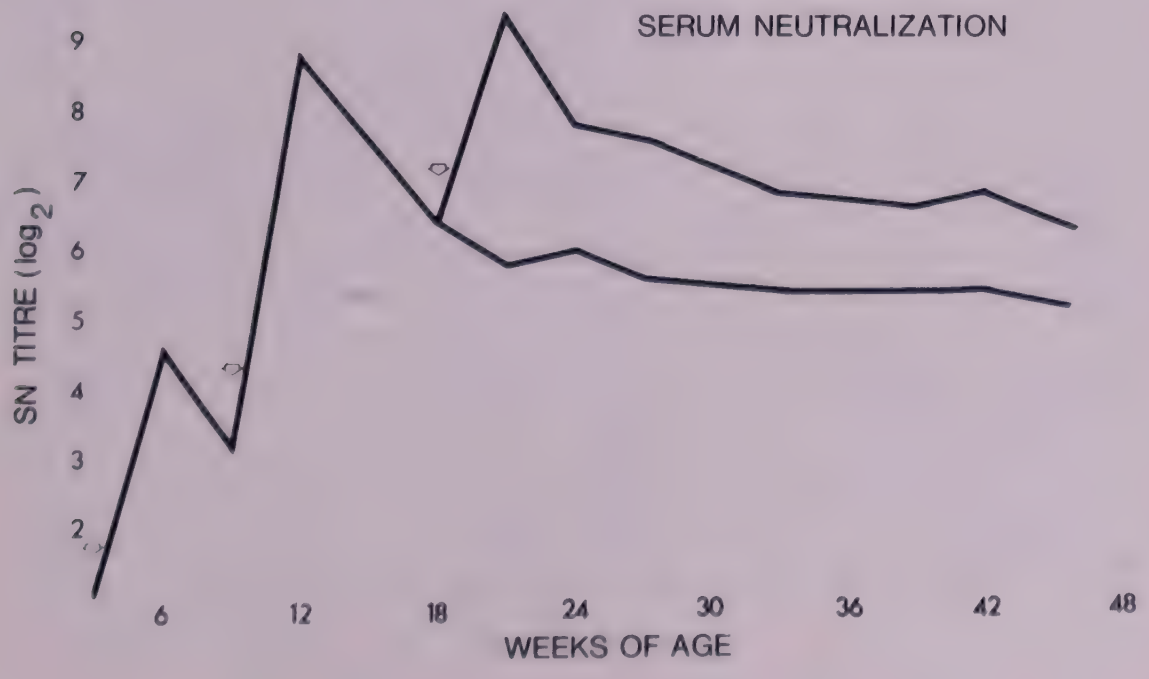
better response than application in the drinking water (dw). Similar results were reported by Benson *et al.* (1975). On revaccination, the milder live vaccines gave a relatively poor response. This was improved when the La Sota strain was used as the boosting agent and improved further when this strain was used by the intranasal route.

In contrast, the use of inactivated vaccine as a booster produced superior responses (Figure 23).

The results illustrate the difference between the primary, secondary and tertiary responses. As in Figures 12 and 25, there is a marked difference between the peak primary and secondary responses. For the attainment of long-term immunity, the plateau which is seen approximately nine weeks after revaccination is maintained at a significantly higher level over a 24-week period, in the case of the tertiary response. The production of a satisfactory plateau level of antibody should be the aim of all vaccination programmes. It is known that live vaccines applied at a dose level between 10^6 and 10^7 EID_{50} per bird depend for their effect on the ability of the virus to multiply in the host. The less virulent the virus, the smaller the degree of multiplication and the less likely the virus is to establish itself in the face of circulating antibody.

In contrast, inactivated adjuvant vaccines containing an amount of antigen equivalent to approximately 10^9 to $10^{9.5}$ ELD_{50} per bird exert their effect simply by contact of the antigen with the lymphoid cells of the bird. The immune response is not dependent on viral multiplication. For this

FIGURE 23. Secondary and tertiary response to K2C inactivated vaccine



reason, inactivated vaccines containing high levels of antigen can be expected to be more potent boosters of immunity than the mild strains of vaccine virus. The virus of live mesogenic vaccines is susceptible to neutralization by circulating antibody, and hence the application of these vaccines has to be timed more carefully than that of inactivated vaccines.

In poultry other than broilers, further boosting of immunity is necessary after 10 weeks of age. The object is twofold: to protect the birds from Newcastle disease during the remainder of the growing period, and to provide, at point of lay, an immune level that will effectively protect the pullets with the minimum of revaccination during the laying period.

Figure 24 illustrates the comparative performance of the mesogenic Komarov strain when injected intramuscularly as a primary vaccine, or as a secondary vaccine following the prior use of the B1 strain. The antibody rise was earlier and slightly greater where the Komarov strain was used as a booster dose. When used alone, the Komarov vaccine resulted in a titre similar to that attained when it was used as a booster.

Figure 25 illustrates the importance of the time interval between primary and secondary vaccination. Four identical groups of birds were vaccinated first with inactivated vaccine at 3 weeks of age and then revaccinated as indicated. The results show progressively higher secondary responses as the time interval between the two injections was increased. Figure 25 also shows the comparative response following the

FIGURE 24. Serological response of birds receiving B1 and Komarov viruses

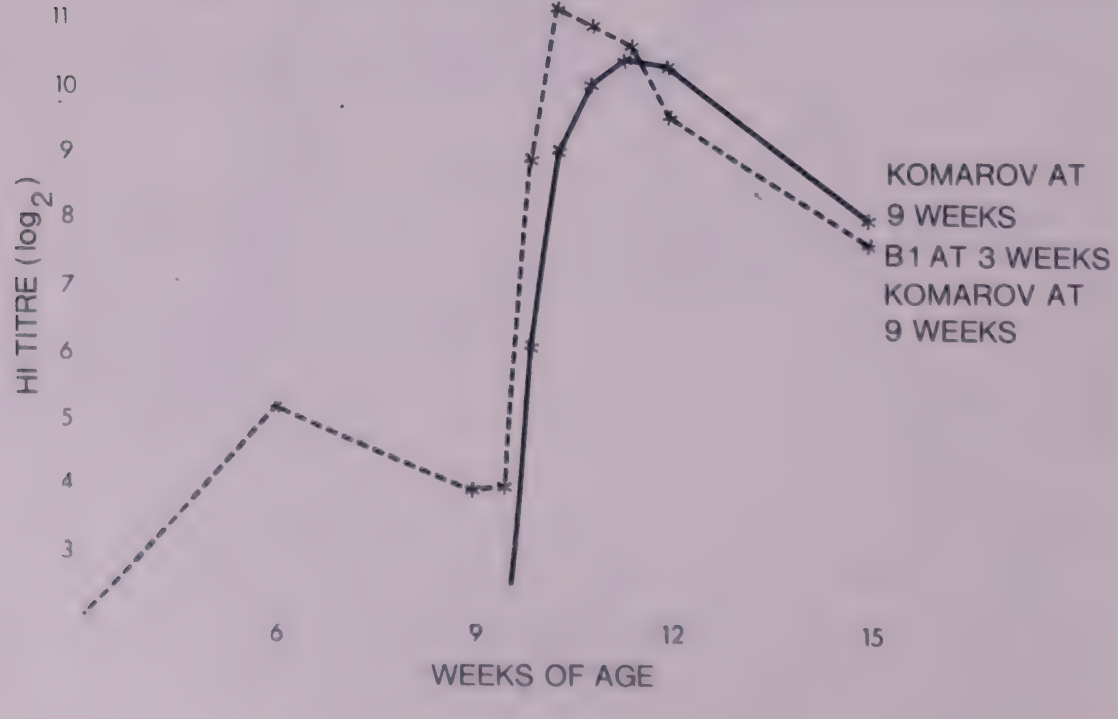
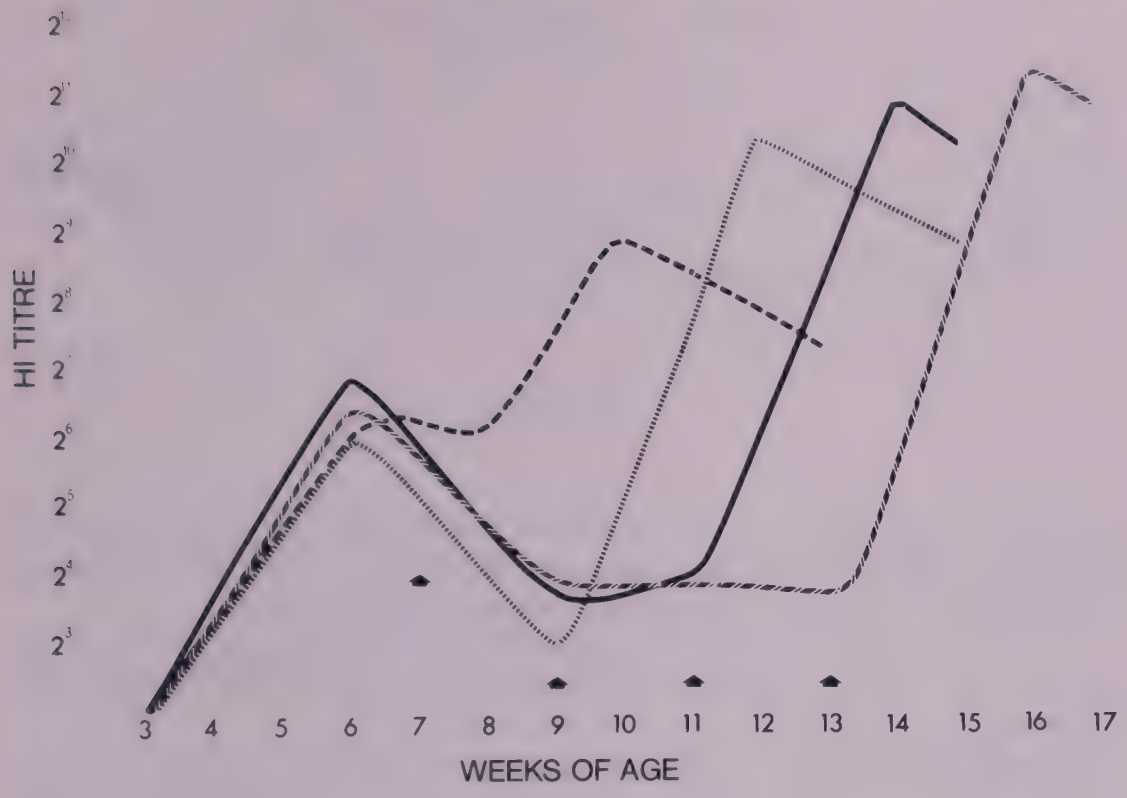


FIGURE 25. Revaccination carried out at varying time intervals with a killed vaccine

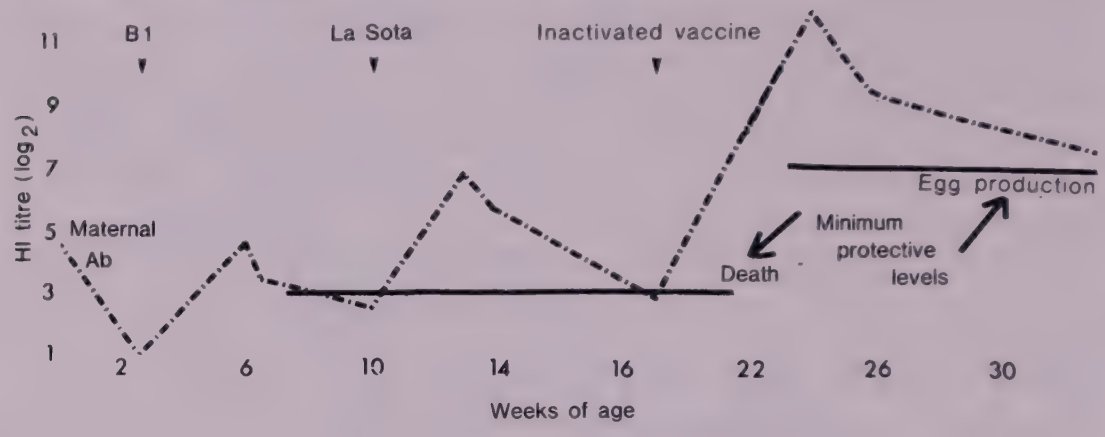


second and third doses of inactivated vaccine. It will be seen that the peak secondary response as measured three weeks after vaccination was about 4 log above the peak primary response. In the succeeding six-week period, the antibody level fell. Following a third dose of vaccine, the peak titre achieved was similar in magnitude to the peak secondary response. The plateau level was increased and formed the basis for the duration of immunity during the egg production period.

On the basis of three successful applications of vaccine, the aim should be to achieve a good level of primary response during the first 35 days of the chick's life. Between 10 and 12 weeks of age this immunity should be boosted by revaccination. An interval during which no further vaccine is given should be allowed until the final dose of vaccine is administered about two weeks before the birds come into egg production.

Figure 26 illustrates a satisfactory vaccination programme. The first vaccination with the B1 strain in the drinking water was conducted at 2 weeks of age when the maternal antibody had disappeared from the majority of the birds in the flock. The primary response resulted in an immune level above that generally needed for protection. At 10 weeks, the stronger La Sota strain was used in the drinking water to produce a longer period of protection.

FIGURE 26. A satisfactory vaccination programme



Before the point of lay, an emulsified inactivated vaccine was used to produce a high level of immunity which could be expected to protect against both the clinical disease and a drop in egg production.

If the eye drop, intranasal or aerosol routes are chosen for the live vaccines, the primary and secondary immune responses will be higher and last longer.

It has been found that control is made more difficult by the persistence, at a low level, of field virus in flocks which are sufficiently well vaccinated to show no overt signs of disease. Therefore, it is difficult to determine when an area is free from virulent virus. High levels of immunity should be maintained for a considerable period after the last case of overt disease, and particular attention should be given to cleaning-out processes. Multi-age production sites are particularly liable to carry the field virus in an occult form. Diagnosis cannot be made on serological grounds, as the activity of the field virus may be so low that few birds show a response that is distinctly higher than a vaccinal response.

To detect latent infection with virulent field virus, SPF " sentinel chickens " have been placed in suspected flocks for one month at the ratio of one " sentinel chicken " per 1 000 birds. Generally, if the flock is infected, the " sentinel chickens " show evidence of field virus within four to seven days (Utterback and Schwartz, 1973).

APPENDIXES

DEFINITION OF TERMS

AAF. Amnio-allantoic fluid.

BPL. Betapropiolactone.

EID_{50}. Embryo-infective dose, 50 percent end point. This is the expression of the virus content of a sample as determined by a dilution series titrated in embryonating eggs, and represents the minimum dose that will infect 50 percent of embryos. The end point may be determined by several mathematical procedures. It is recommended that the Spearman Karber method be used. Where repeated titrations are made, it is possible to express the accuracy of the end point by including the confidence limits following analysis of several titrations. The infectivity of the preparation for any one egg is determined by the demonstration of viral specific haemagglutinin, irrespective of whether the embryo dies or not.

ELD_{50}. Embryo-lethal dose, 50 percent end point. This represents the minimum dose that will kill 50 percent of embryos. It is determined as for EID_{50}, except that the result in any one egg is taken as the death of the embryo by specific virus. The term EID_{50} is used for lentogenic virus and the term ELD_{50} may be used for velogenic virus. It is permissible to use the term EID_{50} in all cases, as it is the wider of the two definitions.

CLD_{50}. Chick-lethal dose, 50 percent end point. This term is used to define the lethal content of a preparation of virulent virus following administration in dilution series to separate groups of susceptible chicks, and represents the minimum dose that will kill 50 percent of the chicks. Interpretation for any one chick is taken as the death of that chick in a manner characteristic of the virus. In the case of contagious virus, it is important to set a time limit on the period of observation, as cross-infection may increase the lethal effect. Birds inoculated with lethal virus usually die within two to seven days following injection. Death is more rapid after injection than after intranasal inoculation or contact exposure.

HA. Haemagglutination.

Harvest. Amnio-allantoic fluid taken from infected eggs after incubation.

HI. Haemagglutination inhibition.

Inoculum. Diluted virus used for infecting embryos.

Production batch. A quantity of virus produced from one inoculum flask. Inoculation, incubation and harvest are all conducted in a consistent manner. If two sources of eggs are used, they should be handled as two different batches.

Seed virus. The quantity of virus divided into aliquots from which dilutions are prepared for manufacture of batches of vaccine. The production of seed virus should be conducted with strict aseptic techniques and full details of the method of preparation recorded. The origin of the seed virus should be described in full, as well as the status of the original material from which it has been prepared (that is, in SPF eggs or in conventional eggs).

SPF. Specific pathogen-free.

VVND. Viscerotropic velogenic Newcastle disease.

RECOMMENDATIONS APPLICABLE TO THE ISOLATION OF POULTRY USED IN THE TESTING OF NEWCASTLE DISEASE VACCINES

1. The poultry facilities should be isolated and geographically removed from the vaccine production laboratory.

2. There must be adequate facilities for incineration. These facilities should be near the isolation area, but remote from the production laboratory.

3. Filtration of all exhaust air is essential if the virulent challenge virus is used, but it is less essential when vaccine virus is used in the isolation facilities.

4. All staff must pass through a shower before putting on clean clothing and boots inside the isolation facilities. The staff must shower and change on leaving the building.

5. Outer protective clothing must be sterilized before laundering. Unsterile protective clothing must be moved from the isolation facilities to the autoclave, using special containers for this purpose. This movement of clothing and containers must be organized in a manner which does not expose the vaccine production area to possible contamination.

6. If the incinerator is also used for the burning of diagnostic material, a routine operation must be established to protect both the production laboratory and the test facilities from possible contamination.

7. If the test birds are likely to carry an appreciable level of Newcastle disease antibody, these birds must be housed in isolation until they are 8 weeks of age. At that time, they must be tested for Newcastle disease HI antibody with negative results. They must also be clinically free from infectious bronchitis and other contagious diseases.

8. The birds used for vaccine testing should all be of standard size. If Newcastle disease-susceptible birds are reared, they should be housed in isolation and at a distance from any domestic poultry, poultry undergoing vaccination tests, poultry on challenge tests, and all post-mortem work.

9. If the challenge procedure is used, all birds must always be incinerated at the end of the test, irrespective of the challenge result. This is very important.

Appendix 3

HIGH MULTIPLICITY OF VIRUS

Table 3-1 illustrates the results of an experiment conducted to determine if the amount of virus inoculated into an egg during a passage technique had any effect on the yield of infective virus. It is known that if embryonated eggs are inoculated serially with high levels of influenza virus, the amount of infective virus produced becomes less and less. This is the von Magnus phenomenon. It is known that the full von Magnus effect does not occur with Newcastle disease virus, but it is not certain if the amount of progeny virus is in any way associated with the dose inoculated. In one experiment the F strain of Newcastle disease virus was passaged in two ways. In the 10^{-1} line, the inoculum consisted of a 1:10 dilution of amnio-allantoic fluid. In the 10^{-5} line, the inoculum consisted of a 1:100 000 dilution. Five eggs were inoculated using each method, and at embryo death the infected fluids were harvested, pooled and subinoculated into the second egg passage in the same manner. At the end of the third passage, the eggs were again harvested, the fluids were pooled and four replicate titrations were carried out on each sample by the fivefold method described in Chapter 7. The results show that the yield of infective virus recovered from the 10^{-1} series was considerably lower in titre than the virus derived from the 10^{-5} series. These results form the basis for the recommendation that an unduly high viral inoculum should be avoided.

TABLE 3-1. — COMPARISON OF THE VARIATION IN YIELD OF F STRAIN VIRUS PASSAGED THREE TIMES IN EGGS AT LOW (10^{-1}) AND HIGH (10^{-5}) DILUTIONS

EID_{50} of virus from third passage	
10^{-1}	10^{-5}
$10^{8.25}$	$10^{8.95}$
$10^{8.05}$	$10^{9.45}$
$10^{8.15}$	$10^{9.25}$
$10^{7.95}$	$10^{9.35}$
mean $10^{8.10}$/0.1 ml	mean $10^{9.25}$/0.1 ml

FREEZE-DRYING

The efficiency with which freeze-drying and storage are carried out will determine the potency of the vaccine under field conditions. The machinery and methods involved in freeze-drying have been described by Rowe (1971). The complete process involves the following steps:

1. Addition of additives to the bulked amnio-allantoic fluids.
2. Dispensing the fluids into vials or ampoules under aseptic conditions.
3. Placing the rubber caps on the vials, leaving space to allow the free escape of moisture from the vials.
4. Loading the vials into the drying chamber of the freeze-drier.
5. Prefreezing the vial contents before vacuum is commenced (this does not apply in the case of centrifugal freeze-drying).
6. Sublimation of water vapour under vacuum (primary drying). See Figure 4-2.
7. Desorption drying under vacuum (secondary drying) to achieve a critical level of dryness. (Note: Overdrying may be as harmful as insufficient drying).
8. Closing the vials either under vacuum or after back filling with a dry inert gas such as nitrogen.
9. Testing for vacuum in the case of vacuum filling (this does not apply to nitrogen-filled ampoules).

Additives

Greaves (1962) has published on the use of additives to give optimal drying conditions. These protective colloids provide a solid matrix to support the vaccine during drying so that moisture can escape and an impervious skin is not formed on the surface of the vaccine plug, prevent excessive dryness from developing, and neutralize a free carbonyl group. This author suggested the use of a solution of glucose-free dextran, 5 to 10 percent sucrose and 1 percent sodium glutamate. Also, dried milk or some other form of protein (e.g., serum or albumen) is usually added (Alboiu *et al.*, 1969; Todorova, 1972; Nedelciu *et al.*, 1973).

Sterile skim milk is an additive commonly used before and after freeze-drying. The amount of glucose or sucrose added to the vaccine will help control the final dryness of the product and will prevent overdrying. However, excess sucrose causes the vaccine plug to have a glass-like surface and prevents the escape of water vapour.

The composition of the vaccine and the additives will determine the eutectic point of the solution. The eutectic point must be determined in order to obtain all constituents of the vaccine suspension in a full frozen state before drying com-

mences. Most freeze driers can prefreeze material to —40°C or lower, but a suspension which has a eutectic point below —25°C should be avoided because freeze-drying is then more difficult. The shape and appearance of the " dried plug " or " tablet "of dried material affect the efficiency of the drying process. A " plug " which separates cleanly from the wall of the vial and which does not form a vapour-resistant skin will freeze-dry quickly and well. Non-viricidal dyes may be added to the vaccine to act as colour codes. It is important that these dyes have no effect on the survival of the virus. Many analine dyes can have marked viricidal properties.

Dispensing the fluids into vials

The volume of vaccine required is determined by the virus content of the amnio-allantoic fluid (AAF) before drying, the dose of virus required and the losses expected during drying and storage. The volume used determines the vial size, and this in turn determines the number of vials that can be loaded on to a tray of the freeze-drier. If raw AAF fluid is purified and concentrated by zonal rotor centrifugation or ultradialysis, then the volume required to provide a given dose may be reduced by as much as fivefold, thus allowing five times the doses of virus to be dried in one load. This may result in considerable savings in time and equipment and in the storing of the finished product.

The amount of AAF or purified virus suspension, together with the additive mixture placed in a 1 000-dose vial, varies from 2 to 5 ml. This is an indication of the large variation in technique existing at present. A production laboratory is advised to regard 2 ml as the minimum volume of raw AAF needed for a 1 000-dose vial. Concentrated and purified suspensions may be reduced *pro rata*. It is preferable to start with a 5-ml amount of virus suspension, and to reduce this when conditions have been developed to allow an effective dose to be produced from smaller quantities of virus suspension.

The vials should be filled so that the liquid is not more than 10 mm deep.

The dimensions in Table 4.1 can be used as a guide.

TABLE 4-1. — VIAL DIAMETER AND VOLUME

External diameter	Volume to give 10-mm depth
..... *mm*	..... *ml* .....
40	10
30	5
22	3
18	2

Working on the basis of 1 000-dose vials, the capacity of the machine is inversely related to the size of the vials used. Hence, if AAF yields are high in virus content and if concentration techniques are used and freeze-drying losses of virus are low, a machine loaded with small-size vials may produce five to 10 times as much vaccine per load as one with large vials of unpurified AAF.

The vials should be filled aseptically in a room adjacent to the drier. The vials should be washed and drain-dried in an inverted position in a drying cabinet at a temperature below 100°C. They should be loaded on to the freeze-drier carrier shelves ready for filling, to avoid subsequent individual handling. The whole shelf unit and vials should be wrapped in aluminium foil or other suitable material and sterilized. A hot air oven at 160°C for one hour may be used, or if an autoclave with a vacuum system to dry the vials is available, they may be autoclaved at 115°C for half an hour. In the former case, it is important to ensure that the vials reach 160°C before the sterilization period is timed. In the latter case, it is essential to follow the autoclave manufacturer's recommended schedule. In both cases, the vials should be allowed to cool to room temperature before opening the sterilizer. Moisture adsorption on to cold vials should be avoided. Chemical indicators and/or bacteriological controls should be included to ensure that the required sterilization temperatures have been reached.

Filling should be carried out from one bulk container using a sterile and carefully calibrated repeating syringe of the Cornwall type. A supply of these syringes fitted with a sufficient length of plastic tubing should be kept sterile and ready for use to be changed quickly if the need arises. It is important that they be accurately calibrated to deliver the correct amount, and the operator should watch for valve failure during the dispensing process because this may seriously affect the volumes measured. Translucent or transparent silicone tubing that can withstand autoclaving should be used. This tubing enables the operator to see any bubbles that enter the system should the tube be accidentally pulled above the level of the vaccine.

Immediately the vials have been filled, silicone rubber stoppers should be placed aseptically and loosely in position. Stoppers sprayed with a silicone solution prior to sterilization and subsequent insertion provide a better vacuum seal when the vials are closed. The design of the silicone rubber seal is important because an adequate depth of rubber at the neck of the vial will ensure longer preservation of dry vacuum during storage.

Similarly, spraying the inside of the vials with a silicone solution before sterilization will help the vaccine " plug " or " tablet " to separate from the glass wall of the ampoule, so that drying of the " plug " is not impeded.

Stoppers should be placed in position with sterile forceps and should be fitted evenly to permit full ventilation of the vial during the sublimation under vacuum. Care must be taken to remove any vials that are inadvertently sealed before the vaccine has dried, because in these cases drying will not be effective. The stoppers should be placed with their top surface approximately horizontal so that at the end of the secondary drying phase all vials can be closed by the pressure plate which will press down on the tray.

Once the vials are filled and fitted with stoppers, they should be frozen immediately.

Determination of the eutectic point

The eutectic point can be defined as that temperature, at atmospheric pressure, at which all constituent fluids of the vaccine suspension are in the solid state. The presence of salts and organic liquids reduces the eutectic point to a temperature substantially below 0°C. Accurate determination of the eutectic point is essential, because without this exact information the maximum temperature at which the vaccine can be sublimed cannot be determined, and melting and refreezing of the suspension during drying may cause serious loss of virus viability.

The eutectic point can be measured on the laboratory bench, but it is determined more conveniently in the drier machine itself by wiring in a resistance controller. The latter equipment is available as an optional extra on most freeze-drying machines, and should always be included when a new drier is ordered. Older machines can easily be modified to include this equipment. The resistance controller consists of a pair of stainless steel probes which are inserted into the bottom of one vial filled with vaccine suspension. The wires are connected to a resistance-measuring device, which should be able to read up to 10 megohms. On many machines this device can be connected to the heater coils of the drying plates so that heat can be cut off immediately the eutectic point is exceeded.

To determine the eutectic point, vaccine in the wired-up vials in the freeze-drying machine should be frozen down to —40°C and then gradually allowed to warm up again to ambient temperature. Resistance and temperatures should be plotted together for both the cooling and warming curves, which will be found to have the sigmoid shape of the examples in Figure 4-1.

This figure shows the rise in electrical resistance with progressive freezing of Newcastle disease vaccine comprising AAF and 3 percent dried skim milk with 0.25 percent sodium glutamate. No plateau of resistance is reached, as would occur with a simple solution; the vaccine continues to increase in resistance reaching a maximum measurement of over —30°C.

The eutectic point should be taken as the top of the log phase of the curve of the warming-up process. With Newcastle disease vaccine, the eutectic point will usually be found at approximately —23 to —25°C, although this can vary greatly with the purity of the solutions used and the nature of the additives.

It is not advisable to set the heater controllers to the exact temperature of the eutectic point, as many heaters will continue to gain heat for several minutes after the current has been switched off. In some cases the increase can be as much as 5°C over the set temperature. With larger machines (with total shelf capacity of 1 m^2 or more), a safety margin of 2 to 5°C can be tolerated. Thus, for a solution with a eutectic point of —23°C, the heater controller should be set not lower than —25°C.

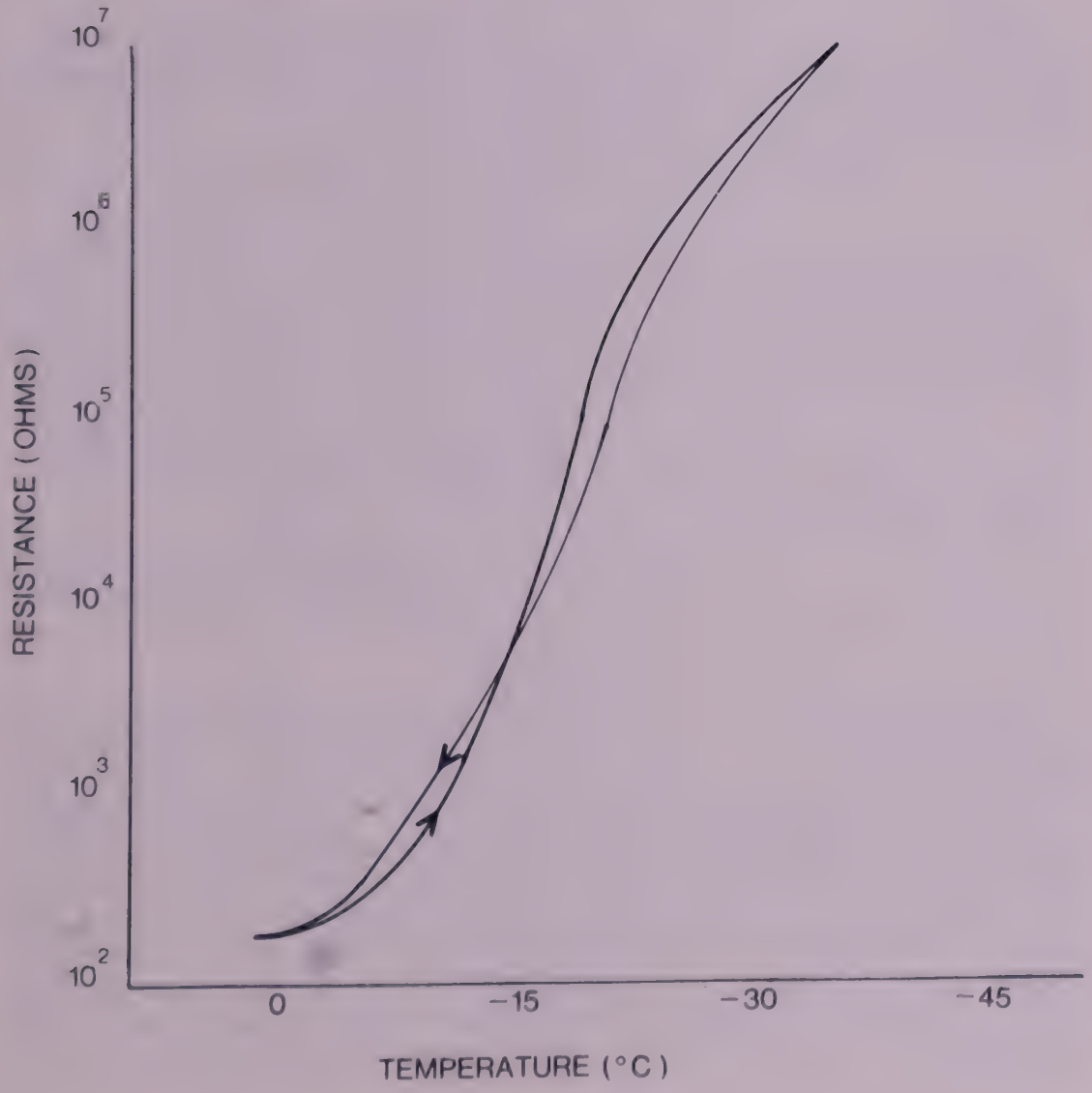
If the freeze-drier is fitted with facilities for continuous cooling of the shelves, the end of primary drying can be detected by a gradual rise in temperature. Secondary drying at 0°C generally presents little difficulty, and in the final stages the cooling coils can be switched off entirely so that the product can reach ambient temperature and be held there for a substantial time before the vials are closed.

Drying at less than —30°C may not be practical because sublimation at this temperature is very slow. If drying is allowed to take place above the eutectic

point, even for a few minutes, the freezing and thawing effects will inactivate progressively more and more virus. It is important that vaccine virus be frozen once only, i.e., during the freezing immediately prior to sublimation under vacuum.

Instantaneous freezing can be obtained by immersing the vials in a mixture of dry ice and hexane during the filling process. The shelf of vials can then be transferred in a well-frozen state to the vacuum chamber. Full vacuum can be applied immediately and heat supplied to bring the vials to just below the eutectic point; they should be held at this point during the whole of the primary drying phase.

FIGURE 4-1. The eutectic point for a Newcastle disease vaccine



Choice of freeze-drier

There are many makes of freeze-drying machines. These should have the extras needed to obtain a high standard of freeze-dried products.

A freeze-drier for vials consists of the following basic components:

1. A chamber of shelves to accommodate the vials.
2. A stoppering device to close the vials at the end of the drying cycle.
3. A condenser to dry the air before it enters the vacuum pump. (Note: Pumps only perform well when pumping almost dry air).
4. A vacuum pump capable of reaching 10^{-2} (0.01) Pascal units under ideal conditions, and operating routinely for several years at a level of not less than 4×10^{-2} (0.04) Pascal units.
5. Freezing facilities for the chamber shelves.
6. Heating facilities for the chamber shelves.
7. Gauges, including vacuum and temperature gauges.
8. A recorder for temperature and pressure.

Additions to the basic freeze-drier should include extra vacuum gauges, additional thermometers, a resistance-measuring device, nitrogen-backfilling facilities and a phosphorous pentoxide secondary drying circuit.

Control of product temperature during primary drying

With a eutectic point that may be as low as — 25°C or lower, it is essential that the freezing capacity of the shelf be able to cool the vaccine as rapidly as possible to significantly below this temperature. It is reasonable to specify that a machine should be able to obtain regular temperatures of — 40°C in the product within two hours, taking into account the ambient temperatures in the production laboratory. When the product can be cooled to this temperature, there is less chance that the vaccine will warm up and begin to " boil " before the vacuum lowers the temperature by sublimation heat loss.

Some laboratories adopt the practice of prefreezing the vials in a low-temperature cabinet prior to placing them on the freeze-drier shelves. However, when this method is used the temperature of the shelves should always be at least — 40°C prior to commencing the vacuum stage.

The condenser should be able to reach — 50°C under all conditions of operation. A condenser that can reach — 60°C is much more versatile and of considerable extra value.

Table 4.2 shows that the vapour pressure of water is markedly altered between — 30 and — 50°C. Figure 4.2 shows the corresponding increased time required for the sublimation of water. At the higher temperature, excess water vapour may bypass the freezing coils of the condenser and seriously affect the ability of the vacuum pump to achieve the required low pressures. When single-stage refrigerators are used, temperatures of approximately — 50°C can be obtained, provided the capacity of the refrigerator is sufficiently large and the expansion valves are well adjusted.

TABLE 4.2. — VAPOUR PRESSURE OF ACQUEOUS VAPOUR OVER ICE

°C								
0	— 5	— 10	— 15	— 20	— 25	— 30	— 40	— 50
mm of Hg								
4.579	3.013	1.950	1.241	0.776	0.476	0.2857	0.0966	0.02955

The quality of a freeze-drier is directly related to the freezing capacity of the refrigerant system, and great care should be taken to ensure that the equipment installed will always operate at a sufficiently low temperature for the purpose required.

The vacuum pump has two tasks to perform. The first is to reduce the vacuum rapidly at the start of operations so that freezing sublimation can commence within 15 minutes after placing the chamber under vacuum. The second function is to maintain a satisfactory vacuum during both primary and secondary drying. These stages may take 48 hours. During the secondary drying stage, the load on the pump is much less, and a pump of smaller capacity is sufficient. It is important that the vacuum pumps be of the two-stage variety, and that they be fitted with an air ballast valve. The function of this valve is to admit small amounts of air to the pump's second stage to allow heating of the interior of the pump and so dry up any collected moisture. The air ballast valve is essential during primary drying when large amounts of water vapour are being removed, and some moisture contamination of the pump is unavoidable. During secondary drying, this air ballast valve should be closed to prevent the pump from heating up unnecessarily.

One manufacturer has recently placed on the market a pump which has the moving parts contained in a replaceable cartridge so that, in the event of undue wear, a new set of sweeping vanes can be fitted quickly and easily by personnel without special engineering skills.

Instrumentation

The machine must have adequate instrumentation. The minimum consists of a vacuum gauge on the backing line of another gauge in the drying chamber. Pirani gauges are the ones of choice but McLeod gauges are also useful. These gauges should read from atmospheric pressure to at least 10^{-2} (0.01) Pascals. Thermocouples are required to determine the temperature of the condenser, the chamber plates and the product. When it can be arranged, each thermocouple should be wired to a separate indicator dial so that all temperatures are readable at the same time. When a selector switch is fitted so that only one temperature can be read at a time, more constant attention to the machine is needed.

It is essential that indicator lights be provided to show when heating and cooling

circuits are closed. Very little critical work can be conducted on a machine which does not have a multichannel recorder to plot the progress of drying. When drying begins, events change so quickly that it is difficult to record them manually. Usually it is not possible for a worker to be present constantly to determine when primary drying is completed and when the ballast valve can be closed, or when the ambient temperatures have been held for an adequate time to complete the drying. Records from the recorder should be filed with the vaccine batch number, and after a reasonable number of batches have been dried, viability tests on the product and the operating conditions achieved should be examined.

A resistance controller which records the rise and fall of electrical resistance in the product is an important accessory, because this is the only means by which the eutectic point can be determined accurately. A resistance controller of this kind also has the function of breaking the heater coil circuits if the product temperature rises too high.

Choice of cooling systems

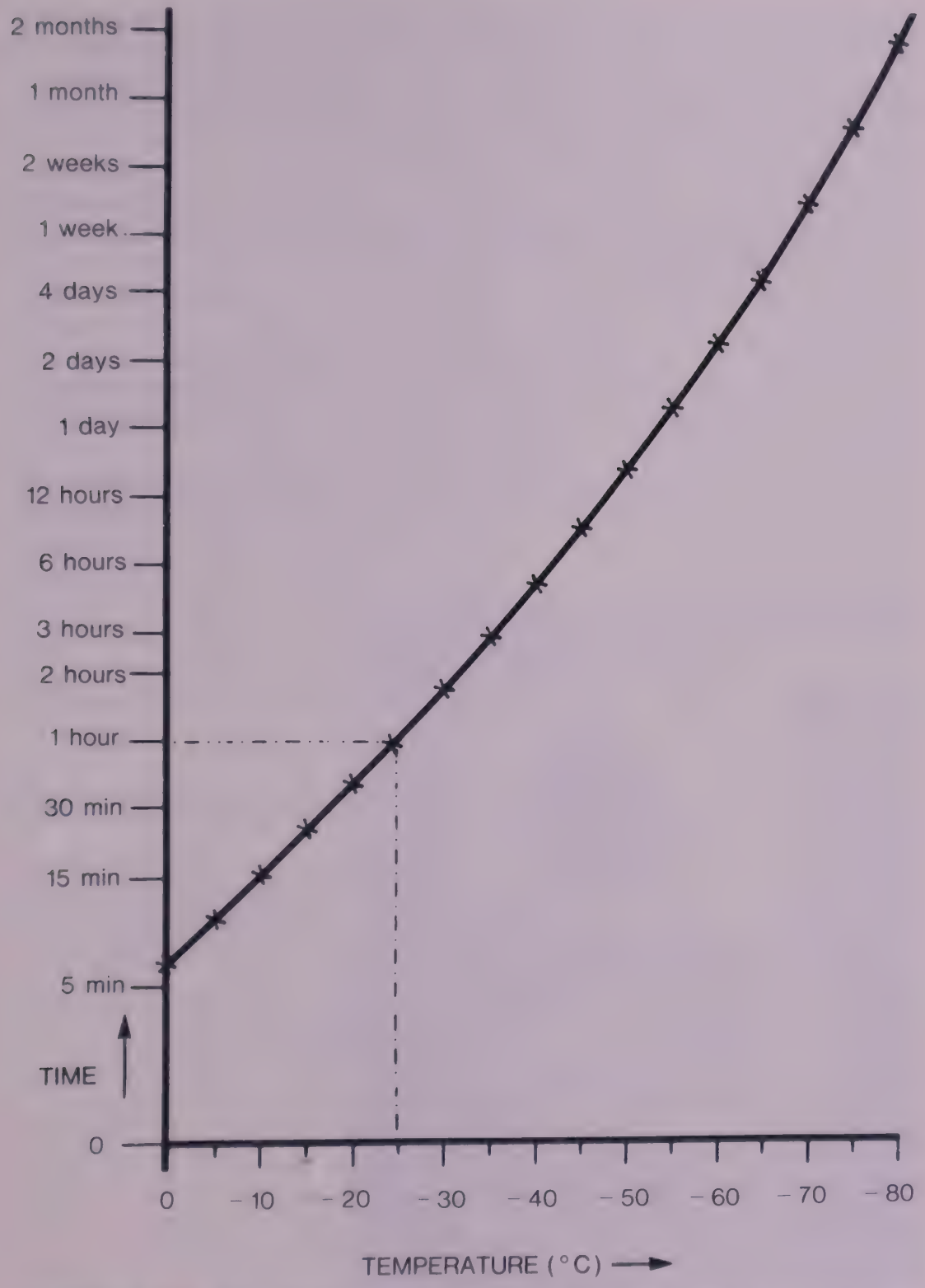
The simplest cooling system is one in which there is a single refrigerator unit which can be used alternately for the condenser, and for shelves, but not for both at the same time. This is the most basic unit. A modification is the same system with a cycling switch which diverts the coolant to either the condenser or the shelves for short periods, thus keeping both at a low temperature. Another modification is the machine with a separate refrigerator for shelves and condenser.

In some of the larger units, shelf temperatures are determined by a circulating fluid which is either heated or cooled in the shelf pipe, so that any predetermined temperature can be maintained at all times. This is the best form of control. The facility to chill the shelves to at least —40°C will allow the drying rate to be fast and safe.

Location of freeze-drying machines

It is important that freeze-driers be carefully located in order to facilitate a good vacuum and refrigeration, easy maintenance and sterility of the product. Large machines are usually built into a wall so that the mechanical side of the machine is in an " engineering " room and access to the shelves and controls is in the clean laboratory area. The exhaust from the vacuum pump should pass through an oil trap and be voided to the atmosphere. Facilities for draining the melted ice from the condenser should be designed with care. A large well-ventilated area should be available for the refrigerator system so that its heat exchanger can operate at maximum efficiency. Air-cooled refrigerators become inefficient if they are operated at a temperature in excess of that recommended by the manufacturer. Machines may also overheat and be switched off by an automatic safety cut-out. For this reason, equipment may fail either to reach or maintain a sufficiently low temperature in the water vapour condenser, or may cease to function altogether. Faults occurring during primary drying may cause the temperature of the freeze-dried product to increase, leading to boiling. During secondary drying

FIGURE 4-2. Relative sublimation times for water



the process may be prolonged, or the product may rehydrate. If local temperatures are likely to exceed 24°C in the room in which the machine is to be located, a large-capacity air-conditioned facility is essential.

Technical expertise required

In addition to virological knowledge, staff members must have sufficient knowledge of refrigerator engineering. They should be trained in vacuum technology and should be familiar with the electrical circuits and the calibration of the thermocouples. It may be advisable that an institute staff member be sent to a freeze-drier manufacturer for comprehensive training in maintenance.

An ample supply of spare parts should always be bought with the machine in order to minimize the time that the laboratory may be inoperative. These spare parts should include a vacuum pump with gauges and thermocouples, and a spare motor for the refrigerator.

Routine checks should be made on the performance of all the freeze-drying equipment.

Where large-scale vaccine production is in operation, it is preferable to have two or three medium-size machines rather than one large machine. This will allow the refitting of a faulty machine to take place without serious disruption of production schedules.

Figure 4.3 shows a modern shelf drier unit that can handle more than 20 000 000 doses a year. Figure 4.4 shows diagrammatically the vacuum system of the machine.

FIGURE 4-3. Shelf freeze-drier

Prefreezing

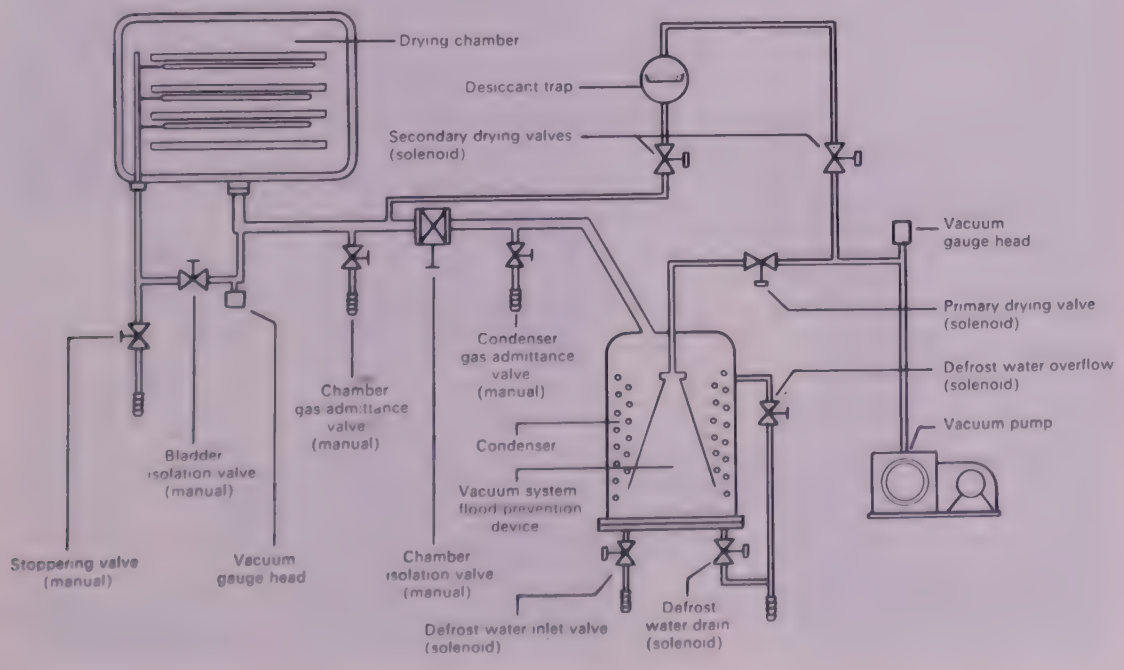
Prefreezing may be carried out in a unit other than a freeze-drier, or in the freeze-drier itself. The latter is recommended because excess movement of filled vials increases the chance of contamination and partial thawing. Freezing on the drier shelves is only efficient if the freeze-drier has the ability to freeze rapidly and reduce the temperature of the contents of the vials to —40°C in a period of about two hours.

The temperature necessary before drying commences is determined by the eutectic point of the virus fluids and additives. A solution based on AAF and sucrose will freeze at about —25°C, thus allowing a considerable margin for warming the product before heat loss due to sublimation takes place.

The changes in temperature and pressure occurring during freeze-drying are shown in Figure 4.6. The process takes a few minutes to stabilize and as the pressure drops by action of the vacuum pump, water vapour passes from the vials to the refrigerated condenser. If the condenser is insufficiently cold and the amount of vapour passing over is large, this vapour may be trapped in the vacuum pump, thus slowing down the vacuum operation. The particular vaccine lot cannot then be dried effectively. The sublimation of water from the vial contents causes the temperature of the vials to drop to about —33°C. If this drop is too great, the sublimation of water vapour will slow dow. To counter this, the shelf heaters are switched on gradually to supply heat so that the drying process can continue. The supply of heat must be monitored to ensure that the vial contents on no occasion reach within 2 degrees of the predetermined eutectic point.

When most of the water vapour has been sublimed from the ice, the shelf

FIGURE 4-4. Schematic diagram of a vacuum system



temperature begins to rise because the loss of heat due to sublimation is beginning to come to an end. The product temperature will then rise either to ambient temperature or toward the temperature set by the circulating fluids in the drier shelf. As the heat exchange between the shelf and the product is by conduction only, the product will tend to be 1 to 2°C warmer than the shelf. A transition from primary drying to secondary drying, during which time the product is held at approximately 0°C for more than two hours, is desirable. Prolonged secondary drying at ambient temperature is needed to reduce the final moisture content to less than 1.7 percent.

It has been shown that with influenza virus excessive drying may lower the viability of the product or give rise to a product which may have fluctuating stability (Grieff and Richtsel, 1968). Any attempt to speed up the process may lead to a poor product. In Table 4.3 it can be seen that under certain conditions a drop of 1 log in titre of influenza virus can occur at + 10°C within as short a time as four days.

Unfortunately, a low temperature in the vial will slow down secondary drying to an unacceptable level. Thus, the time and temperature requirements for secondary drying are a compromise between the time allowable and an acceptable drop in titre. The drier the product, the more heat it can safely withstand, and during the change to secondary drying the temperature should be brought up slowly step by step over a period of several hours to a maximum of 20°C.

FIGURE 4-5. Loading a shelf freeze-drier

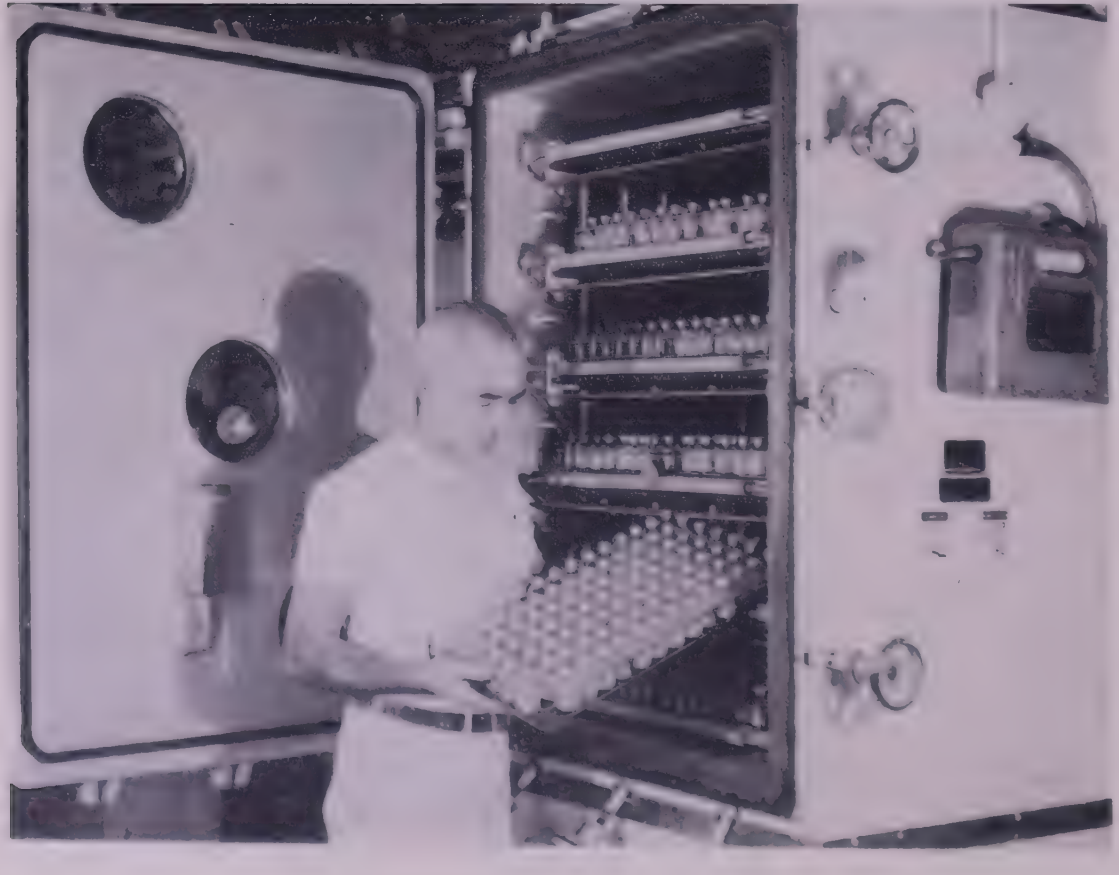


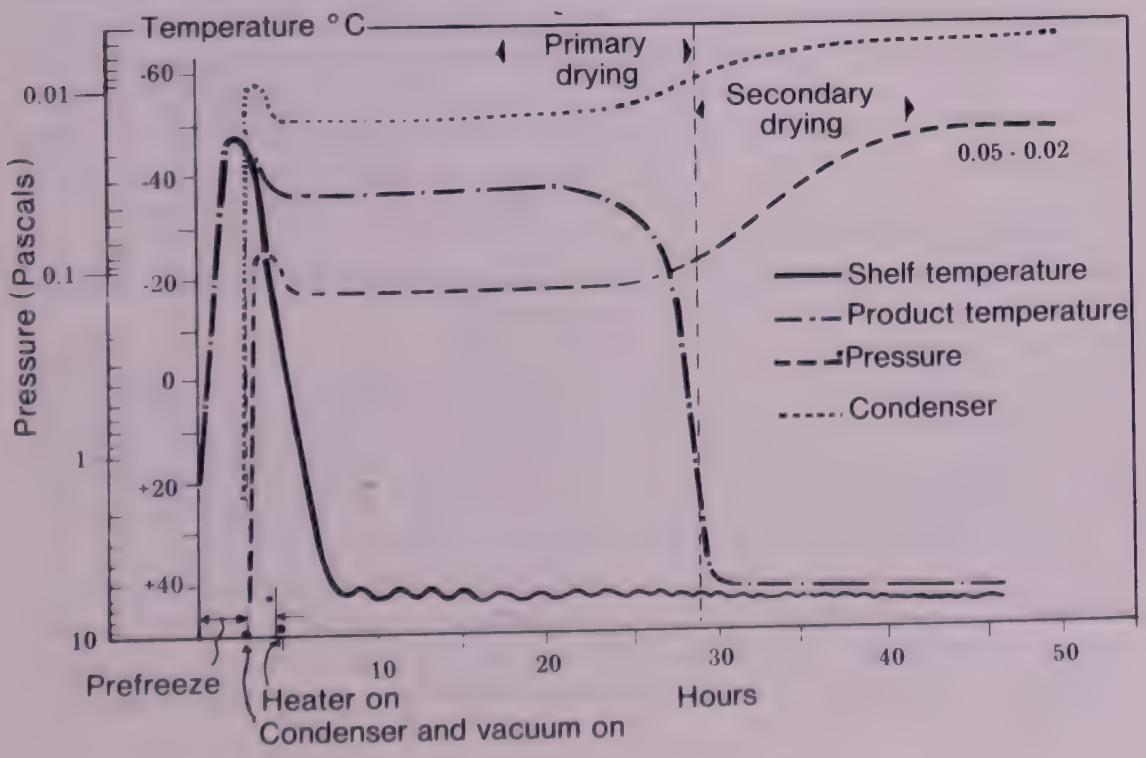
TABLE 4-3. — RELATION BETWEEN RESIDUAL MOISTURE AND DECAY RATE AT VARIOUS TEMPERATURES IN FREEZE-DRIED INFLUENZA VACCINE

Residual moisture	Predicted time to lose 1 log			
	10°C	0°C	—10°C	—20°C
Percent	*Days*			
0.4	4	6	10	16
3.2	7	12	22	40
1.0	24	54	135	480
2.1	68	200	640	1 000
1.7	145	520	1 000	1 000

Completion of secondary drying

The final moisture content of the vial depends on an equilibrium being reached between the vapour removal and the water-binding property of the final product. The completion of the process is indicated by the demonstration of this equilibrium. The pressure in the drying chamber gradually reduces toward the limit of the machine, 4×10^{-2} to 2×10^{-2} (0.04 to 0.02) Pascals. If the chamber is isolated during primary drying, the pressure will rise within a minute to the average vapour

FIGURE 4-6. Typical performance parameter of EF6 shelf freeze-drier



pressure of the vial contents. If, however, there are slight leaks in the vacuum chamber, the gradual rise in pressure may be due to these leaks and not to the increase in partial vapour pressure.

Freeze-drying, storage and virus content

These three items are interlinked. Unpublished research has shown that the relationship between stability of vaccines stored at +4°C and stability of vaccine on an accelerated stability test at 37°C will vary for each type of stabilizer suspension used. Therefore, published data should be interpreted with caution, and vaccine producers should compile their own data. Tests on the international standard for Newcastle disease vaccine (Frerichs and Hebert, 1974) have shown that this preparation can remain stable for seven years when stored at — 20°C and for up to two years when stored at +4°C. A significant loss of titre was found after storage at +37°C for one year. With many commercial vaccines, the decay in viability at +37°C may be in excess of 2 log in a period of less than one week. The work of Frerichs and Hebert (1974) does not confirm the suggestion by Grieff and Richtsel (1965) that degradation is a fully linear process. Studies have shown that degradation is a function of the stabilizer used and the conditions under which drying took place. Current unpublished information suggests that vaccine which is found to be significantly unstable at +37°C is less likely to maintain its titre for long periods at lower temperatures, and the accelerated stability test of +37°C for one week is likely to become generally accepted.

Present evidence suggests that moisture content is critical and that the driest preparation is not necessarily the best. Most preparations can be expected to conserve well, but at higher temperatures the stability may be highly variable.

Residual moisture determination has been based on the modified Karl Fisher technique. More recently, a method has been developed by which the moisture is determined by gas chromatography using a standard preparation of methyl alcohol to dilute the vaccine (Restuccia, 1972). This method is rapid and accurate.

It is important to estimate the decay rates of the vaccine and stabilizer. A 1-log drop over 30 days at +25°C may be expected to be equivalent to a 1-log drop at 0°C over 520 days, and at — 20°C over 1 000 days. The stability of a vaccine is dependent not only on the quality of the dried plug, but also on the air- and moisture-tightness of the silicone rubber vial stopper and the degrees of temperature fluctuation which may occur during storage.

A vaccine which has been well prepared may be expected to drop less than 1 log during storage at +4°C for one year; hence, if at the time of preparation the vaccine contained 7 log per bird dose, at the end of the year it will still contain at least 6 log per bird dose. If storage is at a lower temperature, the loss in titre may well be negligible and it is strongly recommended that bulk supplies be stored at —20°C whenever possible. A stable temperature is also required during transit of vaccine from the central store to the farm, and this should always be arranged to prevent the vials from increasing to a temperature in excess of +20°C for more than a few hours. Handling of vaccine at excessively high temperatures immediately prior to application may be one of the most common causes of vaccination failures.

LABORATORY RECORD SHEETS

SEED VIRUS PRODUCTION SHEET

Material : Freeze-dried vial from _____
marked _____ received _____
Diluted to _____ 10 ___ in _____
Volume of inoculation : _____
Route of inoculation : _____
Source of eggs : _____
Quality of eggs : _____
Number of eggs : _____
Age when inoculated : _____
Incubation period : _____
No. of eggs harvested : _____
Volume of fluid harvested: _____
Titre of AAF : _____
Freeze-dried as _____ x ____ ml ____
Stored at _____ x ____ ml ____

BATCH PRODUCTION SHEET

Page No. _____
Batch No. _____

No. eggs received _____ Source _____ Status _____
No. eggs rejected _____ No. inoculated _____
Seed virus lot _____ Dilution _____ Diluent _____
Volume inoculation _____
Incubation time: _____ for _____ eggs
_____ for _____ eggs
_____ for _____ eggs
Harvest volume _____ ml
Volume rejected at harvest _____ = _____%
Titre of pooled AAF = 10 _____/0.1 ml

_____
Date inoculated

_____ _____
Date harvested Operator

PRODUCTION RECORD SHEET

Page No. _____

Batch No. _____ No. of flasks _____

Blood agar sterility test:

contaminated and rejected _____

not contaminated _____

EID_{50} of predrying sample 10 _____ /0.1 ml

EID_{50} content of sample vials after freeze-drying:

a) _____ /0.1 ml

b) _____ /0.1 ml

c) _____ /0.1 ml

Mean freeze-dried loss _____

No. of vials freeze-dried _____ No. rejected _____

_____ _____

Date freeze-dried Operator

Appendix 6

CONTROL TESTS FOR THE PRODUCTION OF AVIAN LIVE VIRUS VACCINES

Source of materials

Chick flocks. The parent flock shall be SPF for avian adenoviruses, avian encephalomyelitis, fowl pox, infectious bronchitis, infectious laryngotracheitis, influenza type A, leucosis, Newcastle disease, infectious bursal disease (Gumboro disease), reoviruses, *Mycoplasma gallisepticum, Mycoplasma synoviae* and *Salmonella* sp. The flock shall be free from Marek's disease. Control requirements may vary according to the facilities available. The tests listed below are those used by the United Kingdom Ministry of Agriculture, Fisheries and Food.

Alternative tests of equal sensitivity may be considered. Full controls are essential for the performance of these tests. Inactivated antigens are obtainable from the Central Veterinary Laboratory, Weybridge, United Kingdom.

The criteria for the initial acceptance of an SPF flock are based on the absence of the infections specified by examination of samples from the entire flock. Demonstration of freedom from avian encephalomyelitis shall be based on an embryo susceptibility test on not less than 10 percent of one day's egg production with a minimum of 50 embryonated eggs.

The continued absence of specific infections in the donor flock is demonstrated by the above tests on serum samples from 5 percent of the flock and 50 embryonated eggs, both taken at random from the flock at monthly intervals. Permanent records of mortality and results of flock tests are kept.

Seed virus strains

The seed virus shall be designated as to strain and origin and be shown to be free from all extraneous agents.

Tests for extraneous microorganisms[1]

In tests 2, 3 and 4, specific neutralizing antiserum against the vaccine virus which does not neutralize any of the other pathogens listed in Table 6.1 may be used.

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[1] Improved standard control methods will soon be available from the International Association for Biological Standardization, Geneva.

TABLE 6-1. — TESTS RECOMMENDED TO CONTROL SPF FLOCKS

Infection	Strains or antigen in test system	Type of test
Adenoviruses	Any avian strain producing a specific reaction	Agar gel precipitin
Avian encephalomyelitis	Van Roekel	Embryo susceptibility
Fowl pox	Any strain producing a specific reaction	Agar gel precipitin
Infectious bronchitis	Any strain producing a specific reaction	Agar gel precipitin
	Beaudette and Massachusetts 41	Serum neutralization
Infectious laryngotracheitis	Any strain producing a specific reaction	Serum neutralization or agar gel precipitin
Influenza type A	Avian influenza virus type A	Group-specific: Agar gel precipitin Strain-specific: HI test with selected Hav type 1 to 9
Newcastle disease	B1 or F strain Any suitable lentogenic strain	Haemagglutination inhibition
Leucosis	RSV (RAV 1 and RAV 2)	Serum neutralization or COFAL
Marek's disease	Any strain containing A, B and C antigens	Agar gel precipitin
Gumboro disease (infectious bursal disease)	Any strain producing a specific reaction	Agar gel precipitin
Mycoplasma gallisepticum	S6 strain	Agglutination
Mycoplasma synoviae	Any strain producing a specific reaction	Agglutination or HI
Reoviruses	Any strain producing a specific reaction	Agar gel precipitin
Salmonella pullorum	Polyvalent antigen	Agglutination
Duck virus hepatitis	Types 1 and 2	Agar gel precipitin
Duck plague (duck enteritis virus)	Any strain producing a specific reaction	Serum neutralization
Duck septicaemia (*Pasteurella anatipestifer*)	Any strain producing a specific reaction	Agar gel precipitin
Other *Salmonella* infections		Cloacal swab or faecal sampling

1. *Tests for fungi, bacteria and PPLO.* The media used shall be capable of supporting the growth of all aerobic and anaerobic microorganisms. Special media shall be used to detect *Salmonella* sp., *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. Vaccines intended for parental administration shall be sterile. Vaccines intended for administration by other methods may have a count of 10 non-pathogenic microorganisms per field dose.

2. *Tests for extraneous agents using embryonated eggs.* The vaccine sample shall be reconstituted to contain 10 times the minimum field dose per 0.2 ml, and inoculated into three groups of 10 chick embryos from an SPF flock as follows:

Ten embryos 9 to 11 days old: 0.2 ml per embryo, into the allantoic sac.
Ten embryos 9 to 11 days old: 0.2 ml per embryo, on to the chorio-allantoic membrane (CAM).
Ten embryos 5 to 6 days old: 0.2 ml per embryo, into the yolk sac.

The eggs are candled for seven days; embryos dying during the first 24 hours are discarded as non-specific deaths, but a test is not considered valid unless it is based on at least six surviving embryos in each group. All embryos which die after the first 24 hours or which survive the seven days shall be examined. A further embryo passage shall be carried out by each route. The vaccine is not satisfactory if any deaths or abnormalities attributable to the vaccine occur.

3. *Test for avian encephalomyelitis virus.* Twenty embryos 5 to 6 days old derived from avian encephalomyelitis-susceptible stock are inoculated into the yolk sac using 0.2 ml containing 10 times the minimum field dose for each embryo. The embryos are hatched and the chicks observed for 10 days. The chicks should remain free from ataxia during the observation period. The vaccine is not satisfactory if less than 60 percent of the embryos hatch.

Alternatively, 10 day-old SPF chicks are inoculated intracerebrally with at least one field dose of vaccine and observed for 21 days. The vaccine is not satisfactory if more than two chicks die from non-specific causes. The chicks should remain free from signs of avian encephalomyelitis during the observation period.

4. *Test for leucosis viruses.* The vaccine sample is inoculated on to chick embryo fibroblast cultures which are known to support the growth of subgroups A and B of the leucosis viruses. The fibroblasts are subcultured at intervals and maintained for a total period of not less than 21 days. The vaccine is unsatisfactory if any evidence of the presence of leucosis virus is found. Control cultures of subgroups A and B of leucosis virus should be inoculated and tested in parallel. Alternatively, the shortened test (Koski *et al.*, 1970) may be carried out.

NOTE Improved standard control methods will soon be available from the International Association for Biological Standardization, Geneva.

INTERNATIONAL STANDARD FOR NEWCASTLE DISEASE VACCINE (LIVE)

Description

The international standard for Newcastle disease vaccine (live) is intended for the standardization of the virus content of vaccines prepared from the Hitchner B1 and similar strains of virus.

This reference vaccine was established in 1967. It was prepared from the allantoic fluid of embryonated hens' eggs infected with the Hitchner B1 strain. The fluid was dispensed into ampoules in 1-ml amounts and freeze-dried. The ampoules were sealed under vacuum. The average weight of dry material per ampoule has been determined as 109.5 mg with a standard deviation of 0.23 percent.

Distribution

The international standard is distributed by the WHO/FAO International Laboratory for Biological Standards, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, United Kingdom. It is available free of charge in limited amounts. If a laboratory needs more than one ampoule every six months, it is expected to prepare its own reference preparation and to calibrate it against the international standard.

Reconstitution

The material in each ampoule may be reconstituted in any convenient volume of a suitable diluent. Care should be taken to ensure that the entire contents of the ampoule are completely resuspended.

National and laboratory reference preparations

National and laboratory reference preparations should be prepared in a stable form. This may be achieved by freeze-drying aliquots of the reference preparation in neutral glass ampoules and sealing them in an oxygen-free atmosphere by fusion of the glass. The ampoules should be stored in the dark at a low temperature, e.g., — 20°C.

The titre of such a reference preparation relative to that of the international standard should be determined by performing a series of comparative titrations in embryonated eggs. Dilutions should cover the range from 100 percent positive

reactions to no reactions, and should be arranged in a logarithmic series with a serial dilution factor of 3× or 4×. Dilutions within the range $10^{-6.0}$ to $10^{-9.0}$ are suggested for titrations of the international standard. At least six eggs should be used for each dilution.

The EID_{50} of the international standard should be at least $10^{7.0}$ per ml; if it is less than this, it may indicate that the test system is unsatisfactory.

The relative titres of the two preparations should be estimated by the standard statistical methods. This calculation should be based on a series of at least three tests.

It is suggested that the titre of a national or laboratory reference preparation be checked against that of a fresh sample of the international standard about once a year.

The International Laboratory for Biological Standards at Weybridge is willing to advise and assist laboratories in providing their own reference preparations.

Assaying routine batches of vaccine[1]

Once a national or laboratory reference preparation has been prepared, it can be used to assay routine batches of vaccine. The reference preparation is titrated along with every batch of tests. This indicates whether the test system is sufficiently sensitive, and defines the amount of virus in the vaccine independently of the test system. Further recommendations are:

1. If the titre of the reference preparation differs by more than 0.5 $\log_{10}$ from that which it gave in the comparative titrations with the international standard, this indicates that the test system is unsatisfactory and the titration should be repeated after the trouble has been corrected.
2. Each national control authority and/or manufacturer should define the minimum titre of virus acceptable.

[1] This standard is to be used for the assay of virus content and is not to be used as a reference preparation for potency tests.

INTERNATIONAL STANDARD FOR NEWCASTLE DISEASE VACCINE (INACTIVATED)

Description

The international standard for Newcastle disease vaccine (inactivated) was established in 1963. It was prepared from the allantoic fluid of embryonated hens' eggs infected with nine European strains of Newcastle disease virus. The fluid was inactivated with formalin and mixed with an equal volume of an aluminium hydroxide suspension containing 2 percent $Al(OH)_3$. The resulting preparation was mixed with an equal volume of a 10 percent aqueous lactose solution (as a protective agent during freeze-drying) immediately before being dispensed. The mixture was distributed into glass ampoules in 8-ml amounts and freeze-dried. The ampoules were sealed under vacuum. The average weight of dry material per ampoule has been determined as 525 mg.

The standard can be used to assay the potency of both formalin and BPL inactivated vaccines.

International unit

The international unit is defined as the activity contained in 1.0 mg of the international standard. For practical purposes it may be assumed that each ampoule contains 525 international units.

Distribution

The standard is distributed by the WHO/FAO International Laboratory for Biological Standards, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, New Haw, Weybridge, Surrey, United Kingdom. It is available free of charge in limited amounts. If a laboratory needs more than one ampoule every six months, it is expected to prepare its own standard and to calibrate it against the international standard.

Reconstitution

The standard should be reconstituted immediately before it is to be used.

The material in each ampoule may be reconstituted in any convenient volume of a suitable diluent. Care should be taken to ensure that the entire contents of the ampoule are completely resuspended.

National and laboratory standards

National and laboratory standards should be prepared in a stable form. This may be achieved by freeze-drying aliquots of the reference preparation in neutral glass ampoules and sealing them in an oxygen-free atmosphere by fusion of the glass. The ampoules should be stored in the dark at a low temperature, e.g., —20°C.

The potency of such a standard relative to that of the international standard should be determined by performing a series of comparative assays. The following method is suggested:

Chicks of the same age, within the age range of 2 to 6 weeks, drawn from an unvaccinated flock known to be free from Newcastle disease, are used. Serum from each bird is examined by the haemagglutination inhibition test for antibodies to Newcastle disease virus; the batch of chicks is accepted only if all sera are negative in this test. The chicks are distributed into groups of 25 chicks each. A minimum of three groups is injected intramuscularly with the international standard, each group receiving one of a series of doses equally spaced on a logarithmic scale. The same number of groups is injected with a similar series of doses of the preparation being calibrated. The doses are chosen to cover the range from 10 percent to 90 percent protection. This can usually be achieved somewhere within the range of 1 to 40 units per chick. If the appropriate range for the strain of birds and the technique being used is not known, a small-scale preliminary trial should be carried out to determine this. A group of at least 10 unvaccinated chicks is kept as a control.

Fourteen days later, each chick is injected intramuscularly with 200 000 ELD_{50} of a virulent strain of Newcastle disease virus. The chicks are observed for 10 to 14 days and deaths are recorded. Any chicks showing signs of paralysis at the end of this are counted as unprotected. At least 90 percent of the control chicks should die.

The potency of the new standard relative to that of the international standard is calculated by the usual statistical methods, and the result is expressed in international units. This calculation should be based on a series of at least three assays.

It is suggested that the potency of a national or laboratory standard be checked against that of a fresh sample of the international standard about once a year.

The International Laboratory for Biological Standards at Weybridge is willing to advise and assist laboratories in providing national and laboratory standards.

Assaying routine batches of vaccine

The method used can be similar to the one described in the preceding section, but the number of chicks can often be reduced.

When it has been demonstrated that the assay method gives satisfactory and consistent results with any particular vaccine, a four-point assay may be used, i.e., an assay with two groups of chicks for the standard preparation and two for the vaccine being tested. If the slopes of the dose-response curves do not vary significantly from assay to assay, a three-point or a two-point assay may be satisfactory.

However, if this is done, it is advisable to perform a four- or six-point assay from time to time to detect any changes in the assay conditions or qualitative changes in the products being tested.

Potency requirements

Although there are at present no international requirements for inactivated Newcastle disease vaccine, it is suggested that vaccines contain at least 200 international units per dose. Oil emulsion vaccines may be produced at a significantly higher potency level than this.

Appendix 9

STATISTICAL ANALYSIS OF RESULTS

Estimation of EID_{50}, its variance and standard error

It is considered that the most reliable technique for the estimation of a titration end point and its standard error is the Spearman-Karber method (Finney, 1964), although other techniques (e.g., Reed and Muench, 1938) have been widely used. The Spearman-Karber method is simple to compute, and has the advantage that an estimate of the error of titration can also be made.

However, this method is valid only when the dilution range employed extends over the whole, or almost the whole, of the 0 to 100 percent infection range. The calculations are simplified if the dilution series are equally spaced logarithmically, and if equal numbers of eggs are used at each dilution level.

If the full range of infection is not covered, it may be preferable to use a method probit analysis (Finney, 1964).

Examples of calculations using the Spearman-Karber method

1. When the number of eggs at each dilution level is constant (n) and all eggs can be included in the calculation.
   The $EID_{50}(m)$ is estimated from the equation:

$$m = x_k + \frac{1}{2}d - \frac{d\Sigma r_i}{n}$$

where x_k = the highest dilution level tested
d = the interval between successive logarithmic doses
r_i = the number of eggs remaining healthy at any one dilution level
n = the number of eggs at each dilution level (constant).

The variance of m, $V_{(m)}$ is derived from the equation:

$$V_{(m)} = \frac{d^2}{n^2\ (n-1)} \cdot \sum \left\{ r_i\ (n - r_i) \right\}$$

and the standard error of m, $S_{(m)}$ is the square root of the variance, i.e.:

$$\sqrt{V_{(m)}}$$

Example 1

Titration result:

Dilution series (reciprocal $\log_{10}$)	7.0	7.7	8.4	9.1	9.8
No. of eggs healthy/total no. of eggs examined	0/7	1/7	2/7	5/7	7/7

Calculations:

(1) $m = 9.8 + 0.35 - \dfrac{0.7\ (0+1+2+5+7)}{7}$

$= 8.65$

(2) $V_{(m)} = \dfrac{(0.7)^2}{7^2(6)} \times \left\{ (0\times7)+(1\times6)+(2\times5)+(5\times2)+(7\times0) \right\}$

$= 0.0433$

(3) $S_{(m)} = \sqrt{0.0433}$

$= 0.2082$

2. Where the number of eggs at each dilution is not constant (e.g., as a result of non-specific deaths), the calculations may become more complex, but the analysis by this method remains valid.
The $EID_{50}(m)$ is estimated from the equation:

$$m = x_k + \frac{1}{2}d - d(\Sigma p_i)$$

and the variance of m, $V_{(m)}$ is derived from the equation:

$$V_{(m)} = d^2 \sum \left(\frac{p_i \cdot q_i}{n_i - 1} \right)$$

where p_i = the proportion of eggs remaining healthy at any one dilution level
q_i = the proportion of eggs infected at any one dilution level
n_i = the total number of eggs recorded at that level.

Example 2

Titration result:

Dilution series (reciprocal $\log_{10}$)	7.0	7.7	8.4	9.1	9.8
No. of eggs healthy/total no. of eggs examined	0/7	1/6	2/7	5/6	5/5

Calculations:

(1) $m = 9.8 + 0.35 - 0.7\ (0 + 0.1667 + 0.2857 + 0.8333 + 1.0)$
$= 8.55$

(2) $V_{(m)} = (0.7)^2 \left(\frac{0 \times 1}{6} + \frac{0.1667 \times 0.8333}{5} + \frac{0.2857 \times 0.7143}{6} \right.$

$\left. + \frac{0.8333 \times 0.1667}{5} + \frac{1 \times 0}{4} \right)$

$= 0.04389$

(3) $S_{(m)} = \sqrt{0.0439}$

$= 0.2095$

The precision of any titration is given by the variance (V_m). A low value for the variance (and consequently a low standard error) indicates a more precise estimate of the true end point.

The value of the standard error $S_{(m)}$ is that it gives a measure of the variability within a particular test, can be used in the estimation of the 95 percent confidence limits of m, and can be used in tests of significance when comparing the results of different titrations.

All the above calculations have been carried out using the reciprocal of the logarithm to base 10 to express the virus dilution. It is generally convenient to express results in this way, but should the actual titre and its 95 percent confidence limits be required, the antilogarithms of each value should be obtained. In this case, the 95 percent confidence limits become geometrically spaced about the titre instead of arithmetically.

Further analysis

Techniques for further analysis are described fully in standard textbooks on statistics.

THE ASSAY OF VACCINE STRAINS

The Central Veterinary Laboratory at Weybridge, United Kingdom, has compared the properties of the La Sota strain of seed virus. The basic criteria used in the selection of a master seed virus include genetic stability, potency, stress index, the ability to immunize in the presence of maternally derived antibody, and the ability to immunize susceptible birds. Other criteria are shown in Table 10.1.

The potency was determined by the CLD_{50} test in which three-week-old susceptible birds were vaccinated intraocularly with $10^4 EID_{50}$ vaccine and challenged in groups of 15 with 10^6, 10^7, 10^8, 10^9 and 10^{10} ELD_{50} of virulent Herts '33/'56 by intramuscular injection. The 50 percent mortality level was calculated by the Spearman-Karber method, and HI titres were determined at 10 days post-challenge.

The stress index was determined by exposing birds to a standardized aerosol produced from a Collison spray. The degree of reaction was recorded in a manner similar to that for the IVPI test.

The ICPI test was recorded at 8 and 12 days. The longer time interval demonstrated slight differences not shown at 8 days. The ICPI and the IVPI tests were the standard tests described in *Methods for the examination of poultry biologics* (National Academy of Sciences, 1971).

The plaque characteristics were determined by growing each virus strain on chick kidney monolayers with an overlay of magnesium and di-ethyl-amino-ethyl enriched agar. Staining and observations were made after four days.

The maternal immune and susceptible tests were conducted using 40 day-old chicks (20 with maternal immunity and 20 susceptible). The chicks were vaccinated by the aerosol route with $10^6 EID_{50}$ of test virus per bird, using AAF in distilled water and 1:5 000 parts dried milk administered by a Humbrol spray. The results were recorded by HI determinations made at 14 days post-vaccination.

The neuraminidase activity, haemagglutinin stability, elution pattern and mammalian erythrocyte haemagglutination were determined according to National Academy of Sciences (1971) and Lancaster and Alexander (1975).

The results obtained from six different substrains of the La Sota vaccine virus are given in Table 10.1. It will be seen that the stress index differs between substrains, although the potency values were uniformly high.

TABLE 10-1. — ASSAY OF SIX SUBSTRAINS OF THE LA SOTA VACCINE VIRUS

Test	Substrain					
	A	B	C	D	E	F
ICPI (8 days)	0.18	0.12	0.37	0.31	0.45	0.69
ICPI (12 days)	0.34	0.42	0.84	0.70	0.92	1.28
IVPI	0.00	0.00	0.00	0.00	0.00	0.00
Plaque diameter (mm) [1]	1.74 ±0.25	1.51 ±0.27	1.76 ±0.46	1.85 ±0.34	1.57 ±0.19	1.96 ±0.28
Plaque quality [2]	C.E.	C.E.	V.H.	C.V.	C.E.	C.E.
Stress index [3]	0.35	0.90	1.10	1.36	0.81	0.80
Collison HI	$2^{6.7}$	$2^{7.66}$	$2^{7.0}$	$2^{6.3}$	$2^{6.8}$	$2^{6.2}$
Maternal immune [4] HI 14 days post-vaccination	$2^{5.68}$	$2^{5.81}$	$2^{5.0}$	$2^{5.33}$	$2^{4.68}$	$2^{4.75}$
Susceptible HI 14 days post-vaccination	$2^{7.31}$	$2^{6.38}$	$2^{6.0}$	$2^{6.27}$	$2^{6.43}$	$2^{6.7}$
CLD_{50}	$10^{9.74}$	$10^{9.74}$	$10^{10.0}$	$10^{8.9}$	$10^{10.0}$	$>10^{10}$
CLD_{50} HI	$2^{4.6}$	$2^{4.48}$	$2^{4.28}$	$2^{3.73}$	$2^{4.14}$	$2^{5.2}$
Haem/neur'ase ratio	1:2.51	1:2.99	1:2.44	1:3.93	—	—
Haemagglutinin (56°C) [5]	IS	IS	IS	IS	IS	IS
Elution time (hours)	>100	>100	>100	>100	>100	>100
Sheep haemagglutination	+ve	+ve	+ve	+ve	+ve	+ve

[1] For standard method, see Appendix. — [2] D = discrete, V = variable, C = clear, E = even size, H = hazy. — [3] For determination of results, see Appendix. — [4] These results can be variable due to parent antibody status. — [5] IS = unstable.

HYGIENE IN THE CONTROL OF NEWCASTLE DISEASE

In a number of regions where there has been a rapidly developing poultry industry, the use of Newcastle disease vaccines alone has not always provided adequate control of the disease. In these situations, satisfactory results have depended on the adoption of measures of isolation, hygiene and vaccination (Lancaster *et al.*, 1975). This appendix reviews the isolation and sanitary procedures being adopted at present.

Spread of the disease

Many of the routes of spread of Newcastle disease have been reviewed by Hanson (1972), Lancaster (1966), Lancaster and Alexander (1975) and others. The main routes of spread are:

Spread by human agency. Dust and faecal contamination of footwear is common, and satisfactory cleaning of rubber soles with deep treads requires time and the use of a stiff hand brush. Studies have indicated that the mechanical dissemination of virus by persons was considered second only to the movement of live domestic and other birds. Spread by human agency can be varied, and includes the activities of vaccination crews, debeaking crews, manure haulers, rendering-truck servicemen, fly and vermin control personnel, feed company servicemen, cull hen buyers, farm managers and many other people.

The wearing of protective boots, coat and hat which are changed or disinfected between farms is a minimum procedure, but cannot be relied upon completely to overcome the risk of virus spread.

The experience of owners of specific pathogen-free poultry flocks or primary breeder flocks has shown that a shower and total changing of clothing are the only effective means of eliminating spread of virus by the movement of farm staff and visitors.

It is common on many poultry sites to have a series of houses close together. Although the staff may be allocated work in different houses, they will congregate during meal breaks unless separate facilities and equipment are provided. On some large sites, the use of farm clothing of different colours, according to work area, reduces cross-contamination due to congregation of personnel.

Supervisory personnel moving from farm to farm by car or van have been identified as a means of spread of virus. One solution to this problem is to limit the movement of supervisory staff to one farm per day and to provide clean protective clothing and boots for use each day. Newcastle disease virus is relatively thermolabile and is easily killed during usual laundering procedures. No disinfectants are

needed. However, rubber boots should be cleaned with a detergent and then immersed in a hypochlorite solution.

Feed deliveries. One system which has been adopted is to locate the bulk feed hopper inside the perimeter wire fence. This allows the hoppers to be filled from the edge of the outer road. The feed is conveyed from the hopper to the poultry houses by gravity or by a long auger pipe.

Vaccination teams. Perhaps the only effective procedure in limiting virus spread is to arrange for vaccination to be conducted by the site manager instead of a mobile vaccinating team. Where this arrangement is not possible, only one farm per day should be visited by the vaccinating team, and a complete change of protective clothing is necessary between farms.

In addition, all equipment used by the vaccinating team should be cleaned and sterilized between farms. However, it is not practical to disinfect the aerosol machines at present available. Running the machine in open air outside the farm, rinsing out the vaccine reservoir with clean water and removing all dust with water are suitable precautions to take before using the machine on another farm.

Catching teams. On infected premises, such teams become heavily contaminated with virus and therefore constitute a major source of spread. If the whole farm or site is to be depopulated in less than five days, little virus multiplication is likely; also there will be no virus multiplication after depopulation.

When crops of poultry are being reduced in numbers or when continuous stocking is practised, catching teams, poultry crates and vehicles become sources of disease spread. This risk can be reduced by adopting the policy of one farm per day, a change of protective clothing each day, and the daily cleaning and disinfection of the transport vehicles and poultry crates.

Multiple age groups. Continuous stocking procedures on one site are associated with higher disease risk than the "all in - all out" principle. The latter method has the advantage that should disease be introduced, it may be eliminated when all birds are removed and the premises cleaned and disinfected.

Many large broiler units comprise a number of individual houses separated by a relatively short distance, so that the whole site may be regarded as a single unit in respect of the spread of Newcastle disease virus. If it is possible to depopulate the whole site within five days, the litter can be removed from the last house to be emptied before the first group of day-old chicks comes on to the premises. This procedure minimizes residual contamination on the site.

If young chicks are introduced before the whole site is depopulated, the disease cycle may be perpetuated. This situation may not be readily apparent because maternal immune levels in the chicks may prevent rapid onset of disease for two and a half weeks. The subsequent appearance of Newcastle disease may erroneously suggest that the infection originated outside the farm.

Similarly, continuous stocking of commercial egg producing farms will permit low levels of permanent infection to be present. This situation may be due to the carrier state in individual vaccinated birds, or may be due to the slow passage of

Newcastle disease field virus within the flock. Thus the farm may be infected without the vaccinated birds showing clinical disease. On the farms, clinical disease usually occurs when point-of-lay pullets are introduced, especially if these birds have not been revaccinated prior to moving.

A large number of birds on a site generally means a number of different age groups. This in turn results in greater opportunity for continuous infection, which may be subclinical.

Table egg packing stations. The need for empty egg trays and crates to be brought on to farms to await filling presents an important means of spread of Newcastle disease virus. Washable plastic egg trays are a great advantage. The identification of wooden egg boxes to the farm of origin and the fumigation of these boxes using formaldehyde are necessary procedures in the overall control of Newcastle disease. In some egg packing stations, changes in working procedures may be necessary to provide the poultry farmer with washed and sterilized egg trays and boxes.

Breeding farms. These farms house the basic genetic material, and the value of the poultry stock is high. This in turn is associated with strict supervision and health control. These control procedures are often facilitated by the small size of individual flocks and quarantine measures imposed on new stock being introduced. Regular vaccination of breeding stock will result in progeny carrying a uniform level of maternal immunity. This in turn will permit effective primary vaccination of the progeny.

Cleaning facilities on the farm. The level of hygiene attainable may be limited by the type and construction of the farm buildings and the general economy of the farm.

Totally enclosed units with a concrete floor provide greater protection from disease than semi-open houses. Enclosed units permit greater control over aerosol vaccination procedures. On most farms the cleaning of the floor, feeders and drinkers is usually carried out efficiently because the personnel involved can see the nature of the work to be done.

Poultry houses based on plywood often have the disadvantage that beams, pipes and electrical wiring are exposed above eye level. These beams and pipes harbour dust, and under these conditions Newcastle disease virus may survive until the next group of birds is introduced (Lancaster, 1966). Other areas which usually require close inspection are the ventilation fans and air ducts. Anterooms adjacent to the poultry pen often accumulate used clothing, uncleaned boots, used equipment and generally excessive amounts of dust.

Action of chemicals on Newcastle disease virus. This subject has been reviewed by Lancaster (1966) and Hanson (1972). In general, all known viricidal chemicals will destroy Newcastle disease virus with reasonable rapidity. However, virus protected by protein may survive the action of the disinfectant. Environmental conditions, particularly warm temperatures and exposure to sunlight, facilitate the destruction of the virus by chemicals. Thus the important principle is that disinfection should always follow physical cleaning, and in conditions of low

temperatures supplemental heat may be necessary for adequate cleaning and disinfection.

The simple phenolic compounds are affected least by organic matter, and for this reason are more suitable for use in combined cleaning and disinfection operations on heavily soiled surfaces (Harry, 1967). Toxicity to man limits the application of certain chemicals, including those which are used in the gaseous form.

Effective disinfection of surfaces in poultry houses can be achieved (Harry, 1961) by generating formaldehyde from 20 ml formalin per 1 m^3 of poultry house (the formalin is added to potassium permanganate in the ratio of 3 parts by volume formalin to 2 parts by weight potassium permanganate); by dispersing 20 ml formalin per 1 m^3 as an aerosol; or by heating 2.6 g paraformaldehyde per 1 m^3. Satisfactory results using the above depend on good roof sealing and complete absence of ventilation in the case of formaldehyde produced by heating paraformaldehyde.

Furuta *et al.* (1976) found that adequate disinfection of a filtered air positive pressure house (FAPP) and its equipment was obtained when formaldehyde gas was liberated from 40 ml formalin per 1 m^3 capacity. Test bacteria and a strain of avian adenovirus were inactivated by fumigation for 24 hours. There was no evidence that disinfection was influenced by horizontal or vertical surfaces.

It should be noted that the amount of formalin used by Furuta *et al.* (1976) was similar to the amounts found by Lancaster *et al.* (1954) to be required for the destruction of *Salmonella pullorum* on the surface of eggs when fumigation was conducted at room temperature.

Newcastle disease virus is more susceptible to the action of alkali than to that of acid. Thus, 2 percent sodium hydroxide, 1 percent liquor *cresolis saponatus* and 3 percent phenol are all effective against the virus. Formalin as a 1 or 2 percent solution in water or as formaldehyde gas, as described above, is an effective means of destroying Newcastle disease virus. Quaternary ammonium compounds have also been used.

Where metal surfaces are to be disinfected, care is necessary because some strongly alkaline disinfectants have a corrosive effect. Some disinfectants have an adverse effect on fabrics, painted surfaces and natural rubber. Information on characteristics can be obtained from the manufacturer. Disinfectants or cleaning solutions which contain surface-active agents also tend to accelerate rusting (Harry, 1977). Iron is attacked by alkalis which may be present in certain chlorine disinfectants.

Carcass disposal methods. This has been reviewed by Zander (1972). The burial pit or tank disposal unit is essential, and must be large enough to take normal mortality. The pit must have a well-fitting lid and must be located where it will not contaminate drinking water supplies.

Disposal of litter and droppings. Dry litter is readily wind-borne, and this may be controlled with a water spray. Dry or damp litter has been spread on arable land and immediately ploughed under.

Bulk liquid droppings or slurry can harbour virulent Newcastle disease virus for

at least 21 days. Storage for longer than 21 days will increase the decay rate of the virus. These facts must be considered when bulk liquid droppings are stored and removed for final disposal.

Airborne spread. In some epidemics of Newcastle disease airborne spread of the virus has occurred, and there is evidence to suggest that under cool moist atmospheric conditions the virus can be air-borne for 1 000 metres. Therefore, the spread of virus between adjacent poultry houses on the same site may be mostly airborne, especially if forced ventilation of the houses is used. However, over larger geographical areas, airborne spread of virus may only account for one third of the disease outbreaks. Thus it has been concluded that intensive poultry production was not the major problem. Only when intensive production was accompanied by an increase in numbers of poultry in any given area was there an increase in the outbreaks of Newcastle disease. Thus consideration has been given by governments to controlling the geographical location of new large poultry units and the distance between these sites.

Airborne spread of virus depends only on the spreading potential or diffusibility of the strain or type of Newcastle disease field virus. Thus the Essex '70 type of virus was discharged in the exhaled air of clinically affected birds at the rate of $10^{3}ELD_{50}$ per 1 000 litres air sampled. In contrast, the more viscerotropic strains of virus yielded a maximum of $10^{1.5}ELD_{50}$ per 1 000 litres exhaled air.

In addition to discharge of virus from the respiratory tract, the more viscerotropic strains result in excretion of virus in the faeces. Virus in faecal material can remain viable for 21 days, and during this period faeces can become dry and air-borne as dust particles.

Spread by rodents and other small mammals. Reports in the literature have indicated that certain mammals, especially cats, are susceptible to infection with Newcastle disease virus and can act as carriers of the virus for several months (Lancaster, 1966). The mammalian host range may be confined to those animals which are more associated with farm buildings. Thus rats may discharge Newcastle disease virus for up to five days. It is likely that virus is also harboured in mice for short periods. The eating of an infected mouse by a cat could, on the evidence at present available, lead to a natural infection developing in the cat.

When a poultry facility has housed an infected flock and is undergoing cleaning and disinfection, rats and mice are driven by lack of food to adjacent farm premises. Thus it is recommended that before an infected poultry house is cleaned, extermination of vermin be conducted before all remaining poultry feed is removed.

Location and size of poultry units

The probability of infection spreading from surrounding farms is influenced by the location of the farm units. Hence in the establishment of new poultry facilities, careful planning is necessary in the location of the facility away from sources of contamination and disease.

It is also important that an existing large poultry unit not be endangered by the

subsequent development of other poultry farms in the immediate locality. National authorities can play an important role in general disease control by controlling under a licensing procedure the location of new poultry units. This is of particular concern where an established poultry breeding farm might be involved.

In respect of Newcastle disease, a considerable amount of virus is discharged in the exhaust air of infected broiler or egg production units of large size.

The location of poultry farms on the side of main roads facilitates the supply of electricity, water, feed, and the transport of the birds. However, locations of this kind expose the poultry to disease contamination from vehicles hauling live poultry to a processing plant or vehicles transporting poultry litter. A more suitable location for a new poultry farm is away from main highways with access by a feeder road only. Isolated situations may involve higher initial costs in building and in service facilities, but these costs can be reasonably expected to be offset by healthier poultry stock and higher productivity.

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